

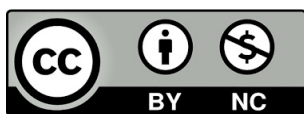
Mariam Bakir Laso

Silver-based nanomaterials as additives in animal feed: analytical methods for the study of their bioaccumulation, transformations and environmental impact

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SILVER-BASED NANOMATERIALS AS ADDITIVES
IN ANIMAL FEED: ANALYTICAL METHODS FOR
THE STUDY OF THEIR BIOACCUMULATION,
TRANSFORMATIONS AND ENVIRONMENTAL
IMPACT

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RESEARCH INSTITUTE OF ENVIRONMENTAL SCIENCES OF ARAGON
DEPARTMENT OF ANALYTICAL CHEMISTRY
UNIVERSITY OF ZARAGOZA

**SILVER-BASED NANOMATERIALS AS
ADDITIVES IN ANIMAL FEED: ANALYTICAL
METHODS FOR THE STUDY OF THEIR
BIOACCUMULATION, TRANSFORMATIONS
AND ENVIRONMENTAL IMPACT**

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DOCTORAL THESIS 2023

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CERTIFY

that this doctoral thesis:

“Silver-based nanomaterials as additives in animal feed: analytical methods for the study of their bioaccumulation, transformations and environmental impact”

has been carried out under her/his supervision by Mariam Bakir Laso in the Group of Analytical Spectroscopy and Sensors of the Institute of Environmental Sciences and in the Analytical Chemistry Department of the University of Zaragoza, to qualify for the title of Doctor in Analytical Science in Chemistry.

and,

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the presentation of this report to be defended to the corresponding Thesis Committee.

Zaragoza, 18 of September 2023

M. Sierra Jiménez García-Alcalá

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*Y cuando la tormenta de arena haya pasado,
tú no comprenderás cómo has logrado cruzarla con vida.
¡No! Ni siquiera estarás seguro de que la tormenta haya cesado de verdad.
Pero una cosa sí quedará clara.
Y es que la persona que surja de la tormenta
no será la misma que penetró en ella.
Y ahí estriba el significado de la tormenta de arena.*

“Kafka en la orilla”. Haruki Murakami

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List of acronyms

AF4	Asymmetric flow field flow fraction
AgNPs	Silver nanoparticles
AMR	Antimicrobial resistance
ANOVA	One-way analysis of variance
AuNPs	Gold nanoparticles
<i>C. reinhardtii</i>	<i>Chlamydomonas reinhardtii</i>
<i>D. magna</i>	<i>Daphnia magna</i>
DLS	Dynamic light scattering
EC₅₀	Half maximal effective concentration
EDS	Energy dispersive spectroscopy
EDTA	Ethylene diamine tetra-acetic acid
EFSA	European Food Safety Authority
ENMs	Engineered nanomaterials
FAAS	Flame atomic absorption spectroscopy
FESEM	Field emission gun scanning electron microscopy
FFF	Field flow fraction
Fabricante	Mobile phase recommended by the manufacturer
HDC	Hydrodynamic chromatography
HPLC	High performance liquid chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectroscopy
ISO	International Organization for Standardization
LA-ICP-MS	Laser ablation coupled to inductively coupled plasma mass spectroscopy
LOD	Limit of detection
LOD_{diss}	Dissolved limit of detection
LOD_{size}	Size limit of detection
LOD_{mass}	Mass per particle limit of detection
LOD_{number}	Number concentration limit of detection
LOQ	Limit of quantification
MALS	Multiangle light scattering
NM	Nanomaterial
NPs	Nanoparticles
<i>O. danica</i>	<i>Ochromonas danica</i>
<i>P. malhamensis</i>	<i>Poterioochromonas malhamensis</i>
PA	DL-penicillamine
PS	Polystyrene
PVP	Poly(vinylpyrrolidone)
<i>R. subcapitata</i>	<i>Raphidocellis subcapitata</i>
ROS	Reactive oxygen species
SC-ICP-MS	Single cell inductively coupled plasma mass spectroscopy
SC-TOF-ICP-MS	Time-of-flight mass spectrometer coupled to SC-ICP-MS
SDS	Sodium dodecyl sulphate
SEC	Size exclusion chromatography

SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SP-ICP-MS	Single particle inductively coupled plasma mass spectroscopy
SSF	Simulated salival fluid
TAP	Tris-acetate-phosphate
t_{dwell}	Dwell time
TEM	Transmission electronic microscopy
TMAH	Tetramethylammonium hydroxide
TSPP	Tetrasodium pyrophosphate
UF	Ultrafiltration

Resumen

En este trabajo se ha evaluado un nuevo nanomaterial basado en nanopartículas de plata metálica (Ag-caolín), utilizado como aditivo promotor del crecimiento en piensos porcinos. Para ello se ha utilizado una plataforma de técnicas analíticas basada en la espectrometría de masas con plasma de acoplamiento inductivo (ICP-MS), utilizada tanto en modo convencional, como de detección de partículas (SP-ICP-MS) y de células (SC-ICP-MS) individuales. Además, se han utilizado técnicas de separación como la cromatografía hidrodinámica (HDC), desarrollando una metodología que ha permitido la detección y cuantificación de especies nanoparticuladas y sus correspondientes especies iónicas, y el fraccionamiento en flujo mediante campo de flujo asimétrico (AF4), ambas acopladas a ICP-MS.

En una primera fase se ha estudiado la bioaccesibilidad de la plata mediante la digestión gastrointestinal *in vitro* de piensos suplementados con el nanomaterial, identificando y cuantificando las distintas formas de plata. Se ha comprobado que la fracción de plata total bioaccesible era alrededor del 8%, encontrándose mayoritariamente en forma de especies solubles de Ag(I), con una pequeña proporción de plata particulada (<0.01%). Estos resultados estuvieron en línea con estudios realizados directamente sobre el nanomaterial en los que se liberaba menos del 40% de la plata en agua, y de sólo un 6% en presencia del pienso, haciéndolo en ambos casos mayoritariamente en forma de especies disueltas de Ag(I). El estudio del comportamiento de patrones de Ag(I) y nanopartículas de plata metálica durante la digestión *in vitro* en presencia y ausencia de pienso, ha permitido confirmar que la matriz afectaba al comportamiento de las especies de plata, reduciendo su bioaccesibilidad en el caso de la Ag(I) del 80% al 8% en presencia de matriz y produciendo la disolución completa de las nanopartículas de plata.

El bajo porcentaje de bioaccesibilidad de la plata se ha confirmado a partir del análisis de diversos tejidos (músculo, hígado y riñón), heces y purines procedentes de un estudio *in vivo* realizado con cerdos alimentados con piensos suplementados con el nanomaterial Ag-caolín. La determinación de plata total en estas muestras ha confirmado que la mayor parte de ésta se eliminaba en heces y purines, acumulándose en hígado y, en menor medida, en riñón, pero no haciéndolo en el músculo.

Debido a que esta plata puede liberarse al medio ambiente a través del uso agrícola de los correspondientes purines, se ha evaluado el posible impacto ambiental desde el punto de vista químico de la plata presente en heces, al no poder disponer de los correspondientes purines, estudiando distintos métodos de extracción. Mediante la lixiviación con agua se ha simulado la movilización medioambiental de la plata, obteniendo una baja liberación (menor del 3%), principalmente, en forma de Ag(I). La extracción alcalina con hidróxido de tetrametilamonio y la extracción secuencial con pirofosfato tetrasódico, amoníaco y sulfuro de sodio han permitido fraccionar las distintas especies de plata presente en las heces para justificar su movilización. La extracción alcalina permitió extraer simultáneamente Ag(I) y partículas de plata, aunque no de forma cuantitativa. La extracción secuencial con pirofosfato tetrasódico, amoníaco y sulfuro de sodio ha permitido diferenciar cuatro fracciones de plata (soluble, partículas metálicas, cloruro de plata y sulfuro de plata) siendo las fracciones mayoritarias las correspondientes a sulfuro de plata (45%) y plata soluble (34%).

Finalmente, se ha evaluado el impacto medio ambiental, desde el punto de vista ecotoxicológico, mediante la exposición de la microalga *Raphidocellis subcapitata* (*R. subcapitata*) a lixiviados del nanomaterial Ag-caolín y heces procedentes de cerdos alimentados con el pienso suplementado. Se ha observado que el aditivo Ag-caolín posee el mismo poder inhibitorio que la Ag(I), mientras que, en el caso de las heces, éste fue menor debido a la presencia mayoritaria de especies de plata poco solubles, lo cual permite considerar que el impacto derivado del uso agrícola de purines procedentes de cerdos alimentados con el aditivo Ag-caolín será bajo.

Summary

In this work, a new nanomaterial based on metallic silver nanoparticles (Ag-kaolin) used as a growth promoter in pig feed, has been evaluated. For this purpose, a platform of analytical techniques based on inductively coupled plasma mass spectrometry (ICP-MS) has been used in both conventional detection mode and individual particle detection mode (SP-ICP-MS) and cell mode (SC-ICP-MS). In addition, separation techniques such as hydrodynamic chromatography (HDC) have been used, optimizing a methodology that has allowed the detection and quantification of nanoparticulate species and their corresponding ionic species; and asymmetric flow field flow fractionation (AF4), both coupled to ICP-MS.

Initially, the bioaccessibility of silver has been studied by the *in vitro* gastrointestinal digestion of the feed supplement with the nanomaterial, identifying and quantifying the different forms of silver. The fraction of bioaccessible silver was around 8%, mostly as soluble species of Ag(I), with a small fraction of particulate silver (<0.01%). These results were consistent with the studies carried out directly on the nanomaterial, in which less than 40% of the silver was released in water and only 6% in the presence of the feed, in both cases, mostly, as Ag(I) species. The study of the behaviour of Ag(I) and metallic silver nanoparticle standards during the *in vitro* digestion in the presence and absence of feed, has confirmed that this matrix affected the behaviour of silver species, for Ag(I), decreasing its bioaccessibility from 80% to 8% in absence of matrix and, for the silver nanoparticles, producing their complete dissolution.

The low percentage of bioaccessibility of silver has agreed with the analysis of the different tissues (muscle, liver and kidney), faeces and slurries from an *in vivo* study with pigs fed with feed supplemented with the Ag-kaolin nanomaterial. The determination of total silver has shown that most of the silver was eliminated by faeces and slurries, accumulating in the liver and, to a lesser extent, in the kidney, but not in the muscle.

Because this silver can be released into the environment through the agricultural use of the corresponding slurries, the possible chemical environmental impact of silver in faeces has been evaluated, since the corresponding slurries were not available, by the study of different extraction methods. Through the leaching with water, the environmental

mobilization of silver has been simulated, obtaining low release values (less than 3%), mostly as Ag(I). The alkaline extraction with tetramethylammonium hydroxide and the sequential extraction with tetrasodium pyrophosphate, ammonium and sodium sulphide have allowed the fractionation of the different species of silver present in faeces to justify their mobilization. The alkaline extraction has allowed the extraction of Ag(I) and silver particles simultaneously, but not in a quantitative form. The sequential extraction with tetrasodium pyrophosphate, ammonium and sodium sulphide has allowed the differentiating of four fractions of silver (soluble silver, metallic particles, silver chloride and silver sulphide), with silver sulphide (45%) and soluble silver (34%) the most abundant fractions.

Finally, the ecotoxicological environmental impact has been evaluated, through the exposition of the microalgae *Raphidocellis subcapitata* (*R. subcapitata*) to the leachates of the nanomaterial Ag-kaolin and faeces from pigs fed with feed supplemented. The additive Ag-kaolin has shown the same inhibitory power as Ag(I), meanwhile, for the faeces, the inhibition was less, due to the presence of poorly soluble silver species, which allowed to consider that the impact derived from the agricultural use of slurries from pigs fed with the additive Ag-kaolin will be low.

I. INTRODUCTION

1. Nanomaterials and nanoparticles

European Commission, in their Regulation number 1169 from 2011, defines nanomaterials (NM) as “a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm. In specific cases and were warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50% may be replaced by a threshold between 1 and 50%” [1]. In a recent recommendation for the NM definition from EC, it was highlighted that the definition of a NM should “be based on the relative fraction of particles in a defined range within the particle number-based distribution of the external dimension of the constituent particles of a material” and “should address materials consisting of particles in solid state, present on their own or bound as constituent parts of aggregates or agglomerates”, among other recommendations, based on the surface or state (non-solid materials should not be considered or large solid products or components), concluding that the definition provided in 2011 had to be updated [2].

In recent years, the use of nanomaterials has increased due to their properties for a wide range of applications [3]. However, they have been used since ancient history, by several civilizations and different periods. For example, ancient Egyptians used lead sulphide nanoparticles 4000 years ago as a hair-dyeing formula. Silver and gold nanoparticles (AgNPs and AuNPs) were used in the Lycurgus Cup, which belongs to the Roman period, to achieve the sensation of colour change depending on the incident light [3–5]. Nonetheless, it was not until 1914 that Richard Adolf Zsigmondy defined the term nanometre and in 1959 the american physicist and Nobel Prize laureate Richard Feynman defined the concept of nanotechnology [3]. In 1974, Norio Taniguchi used for the first time the nanotechnology term to represent the manipulation of the NMs [4]. Nowadays, NMs are used for a wide range of applications, such as food containers, food additives, animal feed, medicine or clothing [6].

According to ISO/TS 80004-2 [7], a nano-object is described as a “discrete piece of material with one, two or three external dimensions in the nanoscale”. Depending on the

number of external dimensions that are in the nanoscale, nano-objects can be classified as nanoparticles, nanofibers or nanoplates. In a nanoparticle, all the external dimensions are in the nanoscale and there are no significant differences between the lengths of the longest and the shortest axes. The nanofiber has "two external dimensions in the nanoscale and the third dimension is significantly larger." The nanoplate only has one external dimension in the nanoscale and the other two are larger.

The most common nano-objects are nanoparticles (NPs). Their size conferred them meaningful properties, including magnetism quantum effects, electrical and high thermal conductivity, mechanical, support for catalysis and antimicrobial properties [3,5]. Thus, the applications of the nanoparticles have grown in recent years, and it is expected that their growth will be increased in the next years [8]. NPs have enormous potential in several fields, such as medicine, catalysis, electronics, alimentation, cosmetics or textiles [9–11]. The increase in their use has caused them to reach the environment. Thus, studying and controlling their toxicity for living beings or their possible environmental risks and ecotoxicity are necessary [12].

As it was said before, nanoparticles can show antibacterial, antifungal and antiviral properties. AgNPs, copper (CuNPs) or selenium (SeNPs) nanoparticles, for example, present these properties [12,13]. They have several mechanisms to carry out this antimicrobial property, such as enzyme inhibition, reactive oxygen species (ROS) generation or disruption of the cell membrane [13]. This is not only important in the medical field but also in the livestock industry for their use as additives.

1.1. Nanomaterials as feed additives

The use of feed additives for animals to improve several factors which are related to animal production (animal efficiency, environmental contamination...) is a wide-extent practice [14]. One of the uses of nanoparticles is, indeed, the supplementation of animal feed [15]. Their small size allows their absorption and better interactions with other feed components when they are ingested orally. They are used to enhance productivity or growth through the improvement of feed quality, digestion and absorption of nutrients, the mineral bioavailability, by their use as carriers; the immune response or intestinal health. They are also used to eliminate antibiotic residues, reduce environmental pollution

and produce animal products free of contaminants [15–17]. Zinc, copper, selenium or silver nanoparticles have been studied as feed additives with different aims. Abdel-Wareth et al. [18] found that with the supplementation of zinc oxide nanoparticles (ZnO-NPs), the improvement of the digestibility of the nutrients and the performance were obtained in broiler chickens, in addition to some biochemical markers of the blood (serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase). ZnO-NPs were also used by Ouyang et al. [19] in weaning pigs, obtaining an improvement in growth and an anti-diarrhoea effect. Nevertheless, in pigs, the addition of ZnO-NPs to the diet of the animal, at low doses, did not improve the growth performance or reduce the diarrhoea occurrence. However, the increase in the dose had a low effect on diarrhoea control [20]. The addition of zinc nanoparticles (ZnNPs) to guinea pig feed by Uniyal et al. [21] achieved a growth improvement and an increase in the antioxidant activity of the enzyme superoxide dismutase. The addition of CuNPs in water for chickens exhibited an increase in immunity and an improvement in chemical blood parameters and the stress-related hormone, decreasing the oxidative stress by the enhancement of the antioxidant potential of the animal [22]. This antioxidant response was also found by the use of SeNPs in lactating dairy cows, which were transferred to the milk [23].

During the 20th century, one of the most used additives was antibiotics. It has been reported that their use caused an increase in nutrient absorption and animal efficiency through the reduction of infections and the increase in the animal weight gain and reducing the feed to weight gain ratio [14]. However, antibiotics can be retained in animal tissues, leading to their consumption by humans, and causing the development of antimicrobial resistance (AMR) [24,25]. For this reason, the European Union forbade their use as feed additives in 2003 (Regulation No. 1831/2003 on additives in animal feed about additives in animal feed) [26]. The limits imposed have made necessary the search for new additives with similar antibacterial properties.

Due to their antimicrobial property and the small dose needed to achieve it, nanoparticles can be used as a substitution for antibiotics as growth promoters [16,18,23,29]. Specifically, AgNPs have been studied to have a positive impact on inhibiting the growth and reproduction of bacteria in animals [16,28–30]. Aksoy et al. [30] saw good antimicrobial activity against the animal pathogens, *Staphylococcus aureus*, *Klebsiella*

pneumonia, *Streptococcus pneumoniae*, *Escherichia coli*, *Bacillus abortus*, and *Mycobacterium bovis*. Fondevila et al. [28] studied the effect of the oral administration of a commercial product called *Argenta*, which consists of silver nanoparticles adsorbed on sepiolite, on weaned piglets, resulting in a reduction of *Coliform*, *Clostridium perfringens* and *Clostridium histolyticum* group in the ileum. An increase in the growth rate, as AgNPs dose increased was also reported. Dung et al. [31] observed an effective antimicrobial effect of AgNPs against the African swine fever virus and *Salmonella* in pig houses.

The mechanisms of the antibacterial activity of AgNPs are four, mainly. They interact with the bacterial cell wall, causing damage to it, increasing its permeability, and leading the cell to death. In addition, inside the cell, AgNPs can interact with bacterial DNA or with other components (such as proteins or lipids), which trigger the creation of ROS, damaging the internal structure of the cell and causing finally the apoptosis of the cell [29,32,33].

2. Detection, characterization and quantification of nanomaterials

2.1. Overview of techniques and methods

The increase in the use of nanoparticles and their possible release into the environment has led to the need to search and develop techniques and methods that allow their analysis. Their composition and morphology affect their fate and behaviour [5,34], so techniques which allow their characterization and quantification are needed. For that, several techniques are available. Nevertheless, the complexity of real samples might be a challenge. In figure 1 [35], the different analytical scenarios related to the analysis of nanoparticles are shown. As it is presented, there are two big groups, one without matrix, which is the simplest scenario (type 0), which corresponds to the analysis of pristine nanoparticles; and the other group, which corresponds to nanoparticles present in samples with matrices of different levels of complexity, from the simplest (type 1) to the most complex (type 3). Type 1 includes consumer and industrial products with NPs in their composition, like textiles or cosmetics. The samples in scenario 2 can be divided into two subgroups, a first one that includes samples from laboratory tests about the fate, release and behaviour of NPs, and a second one involving toxicological and ecotoxicological tests. Finally, type 3 includes the occurrence of nanoparticles in the most complex samples such as food, environmental and organisms [36]. In parallel with the increase in matrix complexity, the concentration of NPs tends to decrease, making more difficult the analysis of the samples [35]. The samples used along this thesis are included in types 2 and 3, mostly.

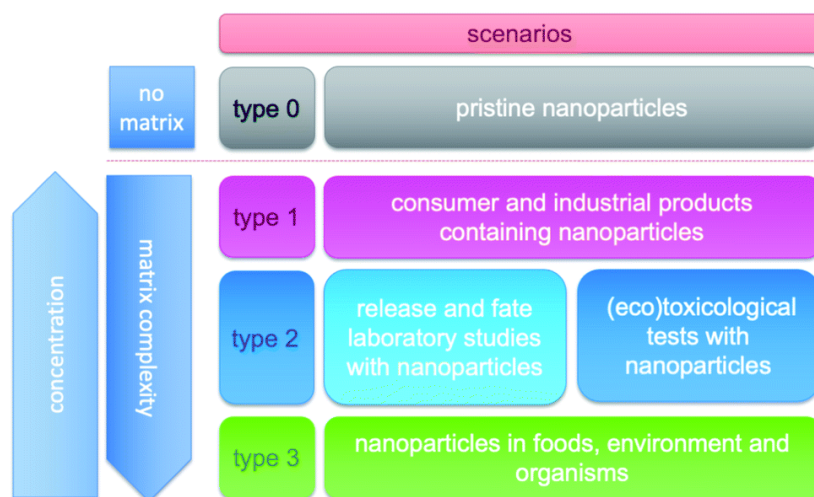


Figure 1. Analytical scenarios related to nanoparticles [35].

Depending on this complexity, different techniques and sample preparation protocols must be used. In the bibliography, several reviews have been published to summarise the most frequent techniques used for the analysis of NMs in different scenarios [37–45]. Mainly, the techniques can be classified into electron microscopies, such as transmission electron microscopy (TEM), field-emission scanning electron microscopy (FESEM) or environmental scanning electron microscopy (ESEM); light scattering techniques, such as nanoparticle tracking analysis (NTA); atomic spectrometry techniques, which include electrothermal atomic absorption (ET-AAS), inductively coupled plasma optical emission spectroscopy (ICP-OES) and inductively coupled plasma mass spectrometry (ICP-MS), in single particle (SP-ICP-MS) or single cell (SC-ICP-MS) modes, laser ablation ICP-MS (LA-ICP-MS) or X-ray absorption spectroscopy; separation techniques, such as field-flow fractionation techniques (FFF), electrophoresis, hydrodynamic chromatography (HDC) and other liquid chromatographic techniques, such as size exclusion (SEC) or reverse phase; electroanalytical techniques, like voltammetry of immobilized particles or particle collision coulometry; and chemical sensors. Depending on the size range of the particles that will be detected, one technique or another shall be chosen (figure 2 [38]). Sample preparation is also important, due to possible interferences with the matrix in the analysis of the nanoparticles. Digestions, dilution, separation, and pre-concentration (centrifugation, ultracentrifugation and dialysis, and liquid and solid phase extraction) are some examples of sample preparation used in environmental samples analysis [37,38]. Bacon et al. [42] completed the review with different sampling techniques (as a System

for the Continuous Aerosol Measurement of Particle Emissions from Roads (SCAMPER) or air samplers) and sample preparation protocols (as microextraction).

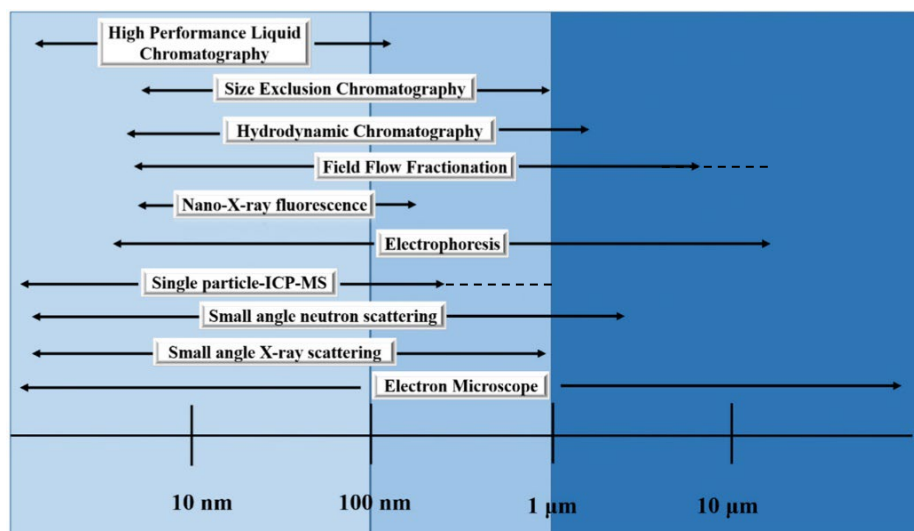


Figure 2. Techniques used for size characterization [38].

In this thesis, samples with complex matrices have been investigated, so it was necessary the development and the utilization of protocols and techniques which allow obtaining information about the nature, concentration and size of the species. For that, several techniques were used: for the total concentration, ICP-MS in conventional mode was used; for the speciation and characterization of the different species, SP-ICP-MS and separation techniques, as HDC and AF4, both coupled to ICP-MS were used. The principal difference between SP-ICP-MS and HDC and AF4 is that with SP-ICP-MS, the mass per particle is obtained and with a mathematical treatment, it can be converted into an equivalent diameter, meanwhile, with AF4 and HDC, the hydrodynamic diameter is acquired. To confirm the size results, electron microscopy techniques (TEM and FESEM) were used. Finally, for the study of the ecotoxicological impact and the internalization of silver by the algae, SC-ICP-MS was used.

2.2. Inductively Coupled Plasma Mass Spectroscopy (ICP-MS)

ICP-MS is an atomic spectrometry technique that involves the atomization and ionization of the analyte in a high-energy argon plasma [40]. It is based on six fundamental parts: sample introduction system, inductively coupled plasma (ICP), interface, ion optics, mass spectrometer (MS) and detector. Liquid samples are introduced in the instrument through

a system that consists of a nebulizer and a spray chamber, where an aerosol of droplets is created. Part of the nebulized sample is transported to a high-temperature plasma, producing ions of the elements which are transferred to the mass spectrometer through the interface. In this mass spectrometer, ions are separated according to their mass-charge ratio (m/z), which are recorded by the detector [46–48].

2.2.1. Single particle ICP-MS

Using ICP-MS at high reading frequencies allows the detection of individual particles. SP-ICP-MS basis was first described by Degueldre et al. [49]. When particle suspensions are nebulized in ICP-MS and are measured with an acquisition frequency over 100 Hz, the particles are recorded as single events above a baseline corresponding to a continuous background and/or dissolved species of the monitored element. The intensity of the particle events is related to the mass of the element in the particle, which can be related to the size of the particles if their composition, density and shape are known or assumed [35,50], obtaining finally a mass per particle or a size distribution (figure 3 [35]).

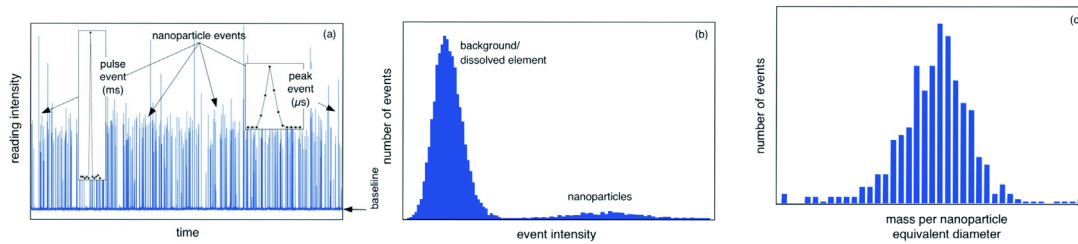


Figure 3. (a) Time scan of suspension containing nanoparticles and dissolved species. (b) Event intensity histogram. (c) Mass per nanoparticle/size distribution of spherical nanoparticles [35].

Determination of the element mass per particle/size and concentration

To obtain quantitative information by SP-ICP-MS about the element mass per particle, size and concentration (both in mass and number), specific data treatment is necessary [51]. In conventional ICP-MS, the mass concentration of a particular element M (X^M), can be related to the signal Y_R as:

$$Y_R = K_R X^M = K_{\text{intro}} K_{\text{ICP-MS}} K_M X^M \quad (1)$$

where K_R is the analytical sensitivity obtained from a mass concentration calibration (counts per second vs. mass concentration), K_{intro} is ($=\eta_{\text{neb}} Q_{\text{sam}}$) and includes the contribution from the introduction system through the nebulization efficiency (η_{neb}) and the sample uptake rate (Q_{sam}); $K_{\text{ICP-MS}}$ is the detection efficiency, which is the ratio of the ions detected versus the atoms introduced in the instrument, $K_M (=A N_{\text{AV}}/M_M)$ is related to the element measured, where A is the atomic abundance of the isotope analysed, N_{AV} is the Avogadro number, and M_M is the atomic mass of the analyte.

When a nanoparticle solution is sufficiently diluted, each recorded event corresponds to a single particle. Under such conditions, the number of particle events (Y_N) during the acquisition time (t_i) is related to the number concentration of particles (X^N):

$$Y_N = K_N X_N = K_{\text{intro}} t_i X^N = \eta_{\text{neb}} Q_{\text{sam}} t_i X^N \quad (2)$$

Where K_N is the analytical sensitivity obtained from a number concentration calibration (number of particle events counted vs. number concentration) and is related to the introduction of the sample and the acquisition time, and it does not depend on the nanoparticles nature.

The net intensity of each particle event S_P can be defined as $S_P = Y - Y_B$, where Y is the gross intensity and Y_B is the mean intensity of the baseline distribution. This parameter is proportional to the number of atoms of the element monitored in each detected particle, therefore, the mass of the element per particle (m_p):

$$S_P = K_m m_p = K_{\text{ICP-MS}} K_M m_p \quad (3)$$

Where K_m is the analytical sensitivity obtained from the mass per particle calibration, plotting the net event intensity vs. the element mass per particle, which depends on the element but is independent of the nebulization process, assuming that the detection efficiency of the particles is the same than for the dissolved element, which may not be the case. If the composition, shape and density are known, the size of the particles can relate to this equation. For solid, spherical and homogeneous particles:

$$S_P = K_d d^3 = \frac{1}{6} \pi \rho F_P K_{\text{ICP-MS}} K_M d^3 \quad (4)$$

2. Detection, characterization and quantification of nanomaterials

where K_d is the slope obtained from the size calibration, where net event intensity vs. particle diameter cubed is plotted, d is the diameter, ρ the density and F_p is the mass fraction of the element in the particle.

And with the following equation, the mass concentration of the dissolved element (X^D) can be calculated:

$$S_{\bar{D}} = K_R t_{\text{dwell}} X^D = K_{\text{intro}} K_{\text{ICP-MS}} K_M t_{\text{dwell}} X^D \quad (5)$$

where $S_{\bar{D}}$ is the net mean baseline signal, t_{dwell} is the dwell time. When dwell times in the microsecond range (200-10 μs) are used, the nanoparticles are recorded as peaks consisting of several readings, whereas at millisecond dwell times nanoparticles are recorded as pulses consisting of one reading.

These equations are fundamental to obtaining different quantitative information. With the event intensity signal, the size (equation (4)) and the mass of the element per particle (equation (5)) can be obtained. The number of events can be also transformed into the number concentration of the particles (equation (2)). Finally, the dissolved mass concentration can be related to the baseline events through equation (5).

In equation (1), the term η_{neb} is introduced. The transport efficiency is defined as the ratio of the amount of analyte that reaches the plasma and the amount aspired [52], and it is needed to determine the number concentration, but also to know $K_{\text{ICP-MS}}$ for the determination of the mass per particle or the size. For its determination, Pace et al. [52] developed two methods: the particle size and the particle number, as alternatives to the conventional waste collection method. The particle frequency method needs nanoparticle number concentration standards to estimate the analyte transport efficiency through equation (2). The particle size method needs dissolved element standards and nanoparticle size (mass per particle) standards to estimate the analyte transport efficiency by the combination of equations (1) and (3).

Alternatively, due to the lack of standards [35] and the possible loss of particles in the tubes and containers, Goenaga-Infante et al. [53] developed a method for the determination of transport efficiency, without the utilization of reference materials. This method is called “dynamic mass flow” and it is based on the gravimetric measurement of

the solvent mass flow with and without including the return of the liquid collected through the waste of the spray chamber. Since the method measures the transport efficiency of the solvent, to be considered as the transport efficiency of the analyte, a cooled spray chamber is required to avoid solvent vaporization.

The matrix of the samples also has to be considered, when the transport efficiency is obtained because the matrix can cause changes in it. For this purpose, the same medium should be used for the calculation of transport efficiency [54].

Determination of dissolved element

Apart from particles containing a specific element, the dissolved element can also be determined if it is present in the suspension. Laborda et al. [55] concluded that information about the dissolved forms can be achieved from the baseline of the time-resolved signal obtained by SP-ICP-MS. This is because the dissolved species are distributed homogeneously in the sample, being nebulized continuously producing a constant baseline, while particles go into the plasma randomly as discrete groups of atoms, producing individual events over the baseline.

An issue to consider in SP-ICP-MS for obtaining quantitative results is the high content of the dissolved element, compared to the concentration of particles, in some samples. When the concentration of the dissolved specie is very high, it can mask the signal of the particles, making it impossible to differentiate between both species [56]. The detection of the particles by SP-ICP-MS is based on its capability to discriminate the particle events, as pulses or peaks, from the baseline, which includes the dissolved element. High contents of dissolved elements increase the baseline, which causes the small particles to be included inside it, not being detected. This causes the underestimation of the concentration of NPs and no actual size would be obtained.

High contents of dissolved specie also cause the necessity of a criterion to discriminate between the dissolved specie and the particles. Gimenez-Inglaturre et al. [57] showed that the use of a $5\sigma_B$ criterion, where σ_B is the standard deviation of the baseline, decreased the probability of having false positives in the complete range of intensities.

In addition, this high content of dissolved species and the small number of particles obtained can deform the size distribution obtained, as well as the concentration in the number of particles [58]. Gimenez-Ingalaturre et al. [57] showed that with the increase of the concentration of the dissolved species (and, consequently, the baseline), the size critical value increased, which led to a reduction of the number concentration value due to the loss of the smaller particles. High contents of dissolved lead in meat caused higher LOD (46 nm and 56 nm) for the analysis of lead NPs (PbNPs), which resulted in incomplete distributions, in which no small particles were visible [59]. The presence of high dissolved contents was also a problem in cellular extracts after enzymatic digestion, preventing the detection of AgNPs of 10 and 30 nm by Malysheva et al. [60]. Temerdashev et al. [61] found that the high background for the analysis of TiO₂-NPs in cosmetic products caused an incorrect estimation of the size of the NPs. They developed a data processing to differentiate the dissolved specie from the nanoparticles with millisecond time resolution. Schwertfeger et al. [62] in a study of soil samples with a high content of dissolved gold (Au(III)), concluded that a minimum of, approximately, 1000 particle events should be achieved to obtain the correct particle size and the right reproducibility. Increasing the acquisition time or diluting the sample may increase the particle events collected. The problem with using a higher dilution is that not only the dissolved concentration decreases but also the particle concentration, which is a problem if the nanoparticle concentration is very low.

Thus, SP-ICP-MS allows the detection and characterization of nanoparticles in samples and allows for discrimination of them from the dissolved species in short times, without complex sample preparation. Nevertheless, in real nanomaterial-containing samples, where the content of dissolved species could be higher, the limitations of SP-ICP-MS made necessary the use of other techniques, such as HDC [63] or AF4 [64].

Meanwhile, the versatility of SP-ICP-MS allows the analysis of a wide range of samples. In recent reviews, the applications of SP-ICP-MS in the analysis of environmental and biological samples have been discussed [35,65–70]. Bolea et al. [35] reviewed the applications of the SP-ICP-MS for the analysis of NPs in the several scenarios presented before (figure 1, page 40), observing an increase in the use of SP-ICP-MS in recent years. A high number of consumer and industrial products, such as cosmetics or food additives,

were reported. The concern about the possible risks for the environment was also highlighted, which translates into an increase in the analysis of samples which are related to the environment, such as the release or fate of nanoparticles in soils, sediments, environmental waters, or ecotoxicological tests. SP-ICP-MS is also used for the analysis of biological samples, such as molluscs, fluids, or tissues. Regarding the environmental applications, Flores et al. [68] considered the use of SP-ICP-MS for the analysis of the leaching of several consumer products (textiles or impregnated products) because this would be a pathway to their reach the environment, as well as the environmental samples themselves (water, air, and soil systems) and in the biological tissues due to their possible accumulation in plants and animals. With the same concern, Rujido-Santos et al. [10] developed a methodology of extraction and subsequent analysis by SP-ICP-MS to detect the release of AgNPs from textiles, with good recovery (around 100%) and validate it with the application to real textiles. In a more recent review, Bakir et al. [65] collected several articles in which the analyte was present in environmental samples (soils, sediments or waters) or came from them, with their release, studying their fate or behaviour. *In vitro* and *in vivo* assays were also analysed, due to their possible toxicity. This toxicity can be caused by the uptake of the nanoparticles by different organisms. SP-ICP-MS allows the study of this uptake with a previous extraction [33,71]. López-Mayán et al. [71] studied the bioaccumulation of TiO₂-NPs in macroalgae by SP-ICP-MS with extraction with TMAH. Nevertheless, the use of SC-ICP-MS, whose basis is similar to SP-ICP-MS, allows the detection of the nanoparticles inside the cells, without the necessity of extraction.

2.2.2. Single cell ICP-MS

ICP-MS in single cell mode has been used for the analysis of unicellular systems. The basis of SC-ICP-MS is similar to the basis of SP-ICP-MS. The technique was applied, first, by Li et al. [72]. They found that bacteria behaviour inside ICP-MS is like large particles and individual events of uranium were visible only when bacteria were present. This means that cells introduced in the instrument are nebulized and vaporized in the plasma, generating an ion cloud of the elements contained. As has been said before, with a high data acquisition frequency, each ion cloud is detected as a single event. The

intensity of the events is proportional to the mass of the element present in the cell, and the number of events is proportional to the number of element-containing cells [73].

For the introduction of the sample, a different spray chamber from the one used by SP-ICP-MS, a conventional double pass of cyclonic spray chambers is usually used for the analysis by SC-ICP-MS. The nebulizers used can be different also, due to the low flow rates used. Different suppliers have developed different sample introduction systems (such as AsperonTM or High Efficiency Sample Introduction SystemTM for spray chambers and MeinhardTM, MassNebTM or Glass ExpansionTM for nebulizers). All of them are based on the same principle. They generate an aerosol with a low-pressure and low-flow nebulizer, avoiding cell damage by using a tangential flow in the linear spray chamber to avoid cell deposition or collisions with the chamber walls [48,74,75].

The low sample preparation needed for the analysis by SC-ICP-MS is an advantage, as well as the low LOD (in the range of fg) achieved [76]. The size, morphology and stability of the cells have to be considered in the analysis by SC-ICP-MS, due to their influence in the transport into the plasma [77]. In fact, the larger sizes of the cells influence the transport efficiency, showing low values (8-13%) for algae, yeast or red blood cells [78].

SC-ICP-MS has been used in the analysis of unicellular systems, like human cells [76,79–82], bacteria [83,84], unicellular algae [78,85–88] or yeast [78,89,90]. Due to the increase in the use of AgNPs, the study of the uptake of silver by cells is necessary to assess their toxicity. Suárez-Oubiña et al. [74] developed a method for the analysis of AgNPs and TiO₂-NPs internalized in cells from sea bass (*Dicentrarchus labrax*) kidney, sea bream (*Sparus aurata*) kidney, and clam (*Ruditapes philippinarum*) gills. They found higher internalization for AgNPs than for TiO₂-NPs, due to the agglomeration of the last ones. Rasmussen et al. [89] analysed the concentration of silver per cell and AgNPs distribution in the cell population in yeast cells. With a washing cycle, they were able to distinguish between the intracellular and the extracellular silver also. Qin et al. [91] determined the uptake of several heavy metals, among them silver, by applying a time-of-flight mass spectrometer to SC-ICP-MS (SC-TOF-ICP-MS), demonstrating that this technique allows monitoring of the changes in metals inside the cells. In human leukemic monocytes (THP-1 monocytes), López-Serrano et al. [76] optimized a method to detect the silver

uptake by the cells. They compared and validated their results with ICP-MS analysis after a digestion process.

2.3. Separation techniques

Due to the limitations of SP-ICP-MS in the analysis of nanoparticles with a high content of dissolved species, it is necessary to complement the results obtained with other techniques that allow the speciation of the different species. For this purpose, HDC and AF4 have been used in this work. Both are techniques based on the separation by size. However, this separation is achieved in diverse ways.

2.3.1. Hydrodynamic chromatography

HDC was developed by Small in 1974 [92]. In that article, the basics of a new chromatography technique based on size was presented. In the following publications, Small developed this technique, improving it [93–95].

HDC is a liquid chromatography technique, which separates particles according to their size. The stationary phase consists of inert non-porous solid beads (polystyrene or silica microspheres) packed into a column. The use of an inert material avoids the interaction of the analyte with the column, which is an advantage of the HDC [96,97].

The separation in HDC is based on the creation of a parabolic or Poiseuille-like profile, developing a laminar flow inside the interstitial space between beads, where the fastest lines are in the centre and the slowest lines are at the lateral. The centre of mass of the larger particles cannot approach the sideways of the column, so they stay in the centre, suffering the fastest streamlines, eluting before the small ones, which can approach the sideways (figure 4) [94,98,99].

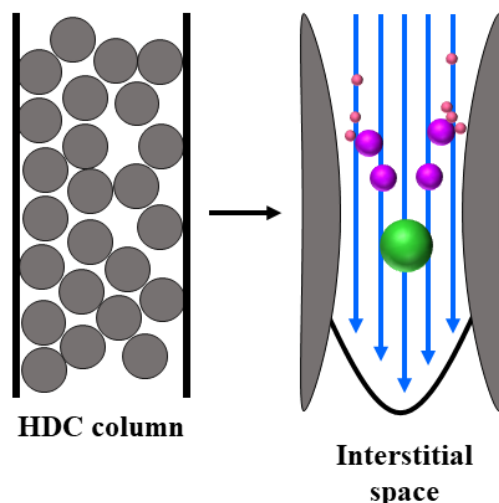


Figure 4. Scheme of the separation basis of HDC.

HDC can be coupled to a number of detectors, like ultraviolet-visible (UV-Vis), dynamic light scattering (DLS), ICP-MS or SP-ICP-MS [37,38]. ICP-MS is most frequently used [96,97,100–105]. With this coupling, better quantitative data was obtained and the speciation between dissolved metal and nanoparticles was possible [100]. Philippe et al. [103] used HDC-ICP-MS to characterize TiO₂-NPs in soils, complementing the information with TEM images. The coupling allowed the determination of the size of the particles and, indirectly, offered information about the composition, in a short time. The coupling to SP-ICP-MS is also reported in the bibliography [104,106–109]. Hachenberger et al. [109] optimized the method to analyse AuNPs in a complex protein-content matrix with HDC-SP-ICP-MS and validated the method. They obtained improvements in the speciation, regarding the measurements by SP-ICP-MS, since they could differentiate between free gold species and loaded by liposomes gold. Roman et al. [106] obtained information about the hydrodynamic size of AgNPs analysed (information provided by HDC) and about the mass distribution (information provided by SP-ICP-MS). The use of HDC-SP-ICP-MS allowed the analysis of samples with low concentrations [104], which can be the case for environmental samples.

HDC, among other techniques, has been reviewed for the analysis of NMs in multiple environmental and biological matrices [38,63,99,110,111]. Real samples containing nanoparticles or spiked ones have been investigated. Philippe et al. [97] developed a methodology based on HDC-ICP-MS to quantify and characterize inorganic colloids in environmental samples. The combination with other detectors, such as UV-Vis and

fluorescence detectors allowed the detection of several elements in matrices with high contents of organic matter. The fate of nanoparticles in environmental waters has been also analysed [108,112]. Chang et al. [108] analysed AgNPs in environmental waters and wastewaters, being able to obtain accurate results for the size of the particles and comparing them with the obtained by DLS. Nevertheless, the interferences caused by microparticles, forced to be centrifuged before the measurements. Proulx et al. [112] also analysed AgNPs in spiked and non-spiked wastewaters, among other NPs by HDC coupled with ICP-MS and SP-ICP-MS, obtaining that with the decreasing of the LOD of the SP-ICP-MS, low concentrations of NPs can be analysed, which allowed the quantification of the NPs in real samples. Changes in the size of NPs, as aggregation and dissolution to form dissolved species were monitored by HDC. Soils were also analysed by HDC-ICP-MS [103] to monitor the size distribution of titanium-containing colloids, providing information about the presence of different compositions and coatings.

In addition, HDC coupled with ICP-MS allowed the separation of NPs and their corresponding dissolved specie. Roman et al. [106] obtained information about the form of silver (Ag(I) and AgNPs) in human plasma and blood, with the coupling of HDC to SP-ICP-MS.

2.3.2. Asymmetrical Flow Field Flow Fractionation

AF4 is the most used technique among field-flow fractionation (FFF) techniques. The difference between AF4 and HDC is the separation mode, in HDC the separation is produced in a packed column as described above, whereas in AF4 an empty channel subjected to a perpendicular flow is used [113].

AF4 was first described by Giddings et al. [114] in 1966, proving that the separation of dissolved macromolecules was possible in a flow FFF channel, with a permeable wall. AF4 is based on the generation of a parabolic laminar flow, which carries the sample from the beginning to the outlet of the channel. Perpendicular to it, there is a physical field which pushes the analytes (macromolecules or particles) to the bottom wall. Once the sample has been injected and focused on the bottom of the channel, the diffusion of the analytes to the upper part of the channel happens due to the Brownian motion. The combination of these two forces causes a size gradient inside the channel which leads to

different elution times according to size (hydrodynamic diameter). In AF4, a perpendicular liquid flow, which is called crossflow, is the force that drives the separation, flowing through an ultrafiltration membrane, placed at the bottom of the channel. A parabolic flow profile is created (figure 5 [115]) and the particles are separated according to their size. The smaller particles will elute before the larger ones [113,115,116].

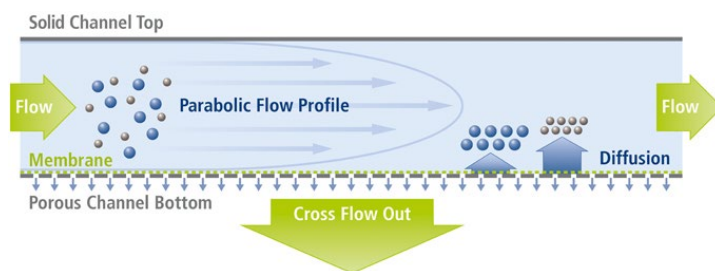


Figure 5. Scheme of the AF4 [115].

The separation in AF4 consists of three principal steps (figure 6 [117]): the focus step, in which the sample is concentrated in a narrow zone of the channel to avoid dispersion through the channel; the elution step, when the diffusion happens, and the particles are separated in terms of their size. During this step, the crossflow pushes the particles against the membrane, meanwhile, the diffusion causes the distancing of the particles from the membrane and enters inside the faster flow. This interaction leads small particles to interact with the fastest parabolic flow. Changes in the flow conditions or the width of the spacer cause changes in the balance of these forces. Although, because of the interactions between the analyte and the membrane, the composition of the membrane and the mobile phase (pH, ionic strength or surfactant) also could affect this balance, and, consequently, the retention time. The resolution and the sample recovery are influenced by these factors, too. The third step occurs when the crossflow is not applied and all the particles which do not separate, elute finally [64,117,118].

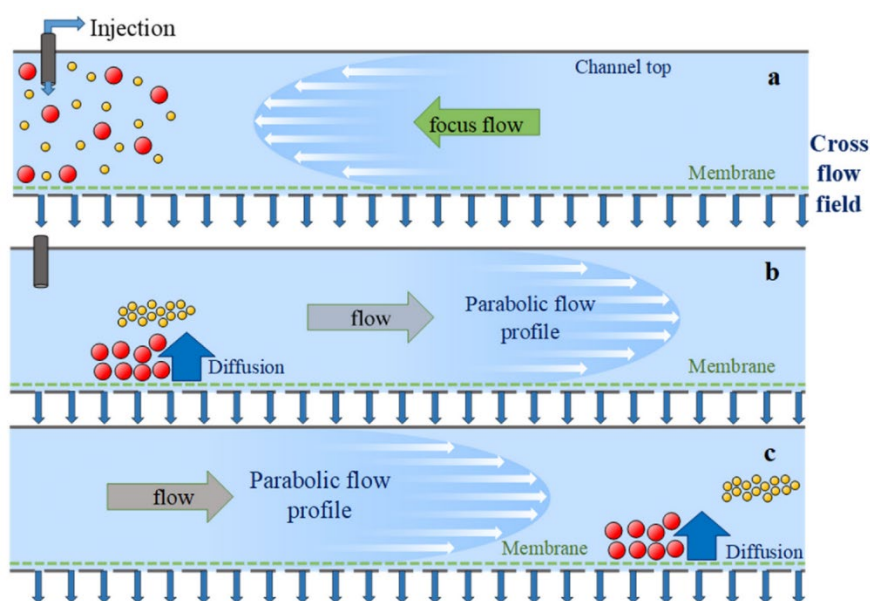


Figure 6. Scheme of the steps of the AF4 separation mechanism [117].

AF4 is coupled to different techniques, to use them as detectors. UV-Vis [119,120], ICP-MS [120–124], nanoparticle tracking analysis (NTA) [125] or DLS [120,126] have been used, although the most common is the combination of several detectors [117,119,127,128].

The use of AF4 for the analysis of nanoparticles in several samples has been reviewed recently [44,124,129–131]. AF4 was used in the analysis of nanoparticles in biological samples [121,132], and environmental samples, such as natural water [133,134]. Worms et al. [124] evaluated the use of AF4-ICP-MS for the analysis of nanoparticles in environmental samples, focusing on the advantages and limits of this technique. AF4-ICP-MS allows the detection of nanoparticles in a wide range of sizes (between 300 Da to 450 nm) with low LOD, which allows their analysis in environmental samples, where the concentration of the nanoparticles used to be low. Nevertheless, the presence of organic compounds makes necessary the improvement of the resolution of the technique by the search for new modes of elution inside the AF4 channel.

Nevertheless, it should be noted that the species with small sizes will be lost through the membrane. This would be a problem with the measurement of dissolved species in complex samples [105,135].

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II. OBJECTIVES

II. Objectives

The main objective of this Doctoral Thesis has been the assessment of a silver-based nanomaterial (Ag-kaolin) to be used as a growth-promoting additive in pig feeding, through the use of a platform of analytical techniques mainly based on inductively coupled plasma mass spectrometry. To fulfil this main objective, several specific objectives were proposed:

- The development of a methodology based on hydrodynamic chromatography coupled to ICP-MS to detect, quantify and characterize gold and silver nanoparticles and their corresponding dissolved species.
- The study of the dissolution/transformations of the silver present in Ag-kaolin nanomaterial in an aqueous medium.
- The study of the bioaccessibility of the silver present in Ag-kaolin nanomaterial by the *in vitro* simulation of the gastrointestinal digestion in pigs.
- The study of the accumulation in tissues (muscle, kidney and liver) and the excretion in faeces and slurries of the silver administered as Ag-kaolin to pigs in an *in vivo* experiment.
- The study of the release of silver from faeces and the fractionation of the silver species present in faeces by applying different extraction methods.
- The study of the ecotoxicity of the additive and other materials derived studied along the thesis, by exposing their leachates to microalgae (*R. subcapitata* and *C. reinhardtii*).

III. EXPERIMENTAL

Capítulo 1. Desarrollo de un método de especiación y cuantificación de nanopartículas de oro y plata y sus correspondientes especies iónicas por HDC-ICP-MS

The content of this chapter was published as *Evaluation of hydrodynamic chromatography coupled to inductively coupled plasma mass spectrometry for speciation of dissolved and nanoparticulate gold and silver* in *Analytical and Bioanalytical Chemistry*, 413, 1689–1699, 2021. (<https://doi.org/10.1007/s00216-020-03132-3>) ([Appendix 4](#)).

Abstract

In this chapter, the development of a method for the separation and determination of metal nanoparticles (gold and silver) and their corresponding ionic species was carried out by HDC-ICP-MS. For this purpose, the mobile phase composition was optimized focusing on the column recovery and resolution between species. Adding penicillamine to the mobile phase, with a concentration of 0.05 mM, allowed the quantification and recovery of Au(III) and AuNPs smaller than 50 nm, while 1 mM was required to recover Ag(I) and AgNPs up to 40 nm. The resolution achieved between Au(III) and 10 nm AuNPs was 0.7, while for Ag(I) and 10 nm AgNPs was in the range of 0.31 to 0.93, depending on the mobile phase used. The detection limits obtained with the optimized mobile phases were 0.05 and 0.75 $\mu\text{g L}^{-1}$ for gold and silver, respectively. The methodology developed has allowed to separate gold and silver species in less than 10 min. The analysis of nutritional supplements containing different species of gold and silver showed results in agreement with those obtained by electron microscopy and ultrafiltration (UF). Due to the resolution obtained, the quality of the quantitative results was affected by the relative abundance of the NPs and the ionic species, as well as by the size of the nanoparticles.

Resumen

En este capítulo, se ha llevado a cabo el desarrollo de un método para la separación y determinación de nanopartículas metálicas (oro y plata) y sus correspondientes especies iónicas mediante HDC-ICP-MS. Para ello, se ha realizado la optimización de la composición de la fase móvil, poniendo especial énfasis en la recuperación de columna y en la resolución entre las distintas especies. La adición a la fase móvil de una disolución de penicilamina con una concentración de 0.05 mM ha permitido cuantificar y recuperar Au(III) y AuNPs con un tamaño menor de 50 nm, mientras que ha sido necesaria una concentración de 1 mM para recuperar Ag(I) y AgNPs con un tamaño menor de 40 nm. La resolución conseguida entre Au(III) y AuNPs de 10 nm fue de 0.7; mientras que para Ag(I) y AgNPs de 10 nm estaba en el rango entre 0.31 y 0.93, en función de la fase móvil utilizada. Los límites de detección obtenidos con las fases móviles optimizadas fueron de 0.05 y 0.75 $\mu\text{g L}^{-1}$ para oro y plata, respectivamente. El método desarrollado ha permitido la separación de especies de oro y plata en menos de 10 min. El análisis de suplementos nutricionales con diferentes contenidos de oro y plata, mostraron resultados que coinciden con los obtenidos por microscopía electrónica y ultrafiltración (UF). Debido a la resolución obtenida, la calidad de los resultados cuantitativos se vio afectada por la abundancia relativa de las NPs y de las especies iónicas, así como por el tamaño de las nanopartículas.

1. Introducción

Como se ha comentado en la introducción, el incremento en el uso de nanomateriales a base de plata en productos de consumo durante los últimos años ha hecho necesaria su detección y caracterización, debido a que, tanto la presencia de formas disueltas, como sus propiedades fisicoquímicas (forma, tamaño o recubrimiento) influyen en su comportamiento y toxicidad [1–4]. Así pues, para entender su impacto ambiental o sus mecanismos toxicológicos, es esencial determinar la morfología (forma y tamaño) y el tamaño de las partículas presentes en una muestra y discriminar entre la especie iónica y sus correspondientes formas particuladas. En numerosos trabajos se han revisado

diferentes técnicas y enfoques metodológicos para la caracterización y cuantificación de nanopartículas y sus derivados en muestras complejas [5–9].

AF4 y HDC son dos técnicas muy útiles para el análisis de nanopartículas. Ambas se basan en la separación por tamaño. Como se ha explicado, mientras que en AF4 la separación se produce gracias a un flujo cruzado que se aplica sobre una membrana porosa, donde se retienen las partículas [10]; en HDC la separación se produce gracias a una columna con un empaquetamiento inerte, que evita su interacción con el analito [11]. Con el acoplamiento a un detector, como un detector UV-Vis o un ICP-MS, se convierten en métodos muy eficaces para la separación, determinación y cuantificación de NPs inorgánicas en una amplia variedad de muestras [5,12]. SP-ICP-MS es otra técnica muy utilizada en la detección y caracterización de NPs. Sin embargo, como ya se ha indicado, una de las limitaciones de SP-ICP-MS se produce con la presencia de concentraciones altas del metal disuelto frente a las nanopartículas. Esto provoca que la intensidad de la línea base sea alta y que las partículas se engloben dentro de ella y, por tanto, no se detecten como partículas individuales, haciendo que su concentración se subestime y el tamaño dado aumente respecto al real [13]. Esto es una desventaja en muestras en las que la concentración del metal disuelto es alta y el tamaño de partícula es pequeño. Chang et al. [14] compararon las técnicas de HDC, AF4 y SP-ICP-MS como técnicas para la obtención del tamaño de partícula, en el análisis de AgNPs en medios acuosos. Obtuvieron finalmente que SP-ICP-MS ofrecía buenos resultados cualitativos y cuantitativos. No obstante, el tamaño de partícula que presenta no es el hidrodinámico, como en el caso de HDC y de AF4. Por HDC obtuvieron mejores recuperaciones que por AF4, a pesar de que su resolución es menor. Gray et al. [10] compararon las técnicas de AF4 y HDC, acopladas a ICP-MS, en términos de resolución, recuperación de columna y LOD, utilizando AuNPs como analito. Concluyeron que HDC es una técnica de separación muy versátil y robusta, pero con un poder resolutivo menor que AF4 para partículas de menor tamaño. Sin embargo, en el caso de la recuperación de columna, AF4 presentó valores más bajos, entre 4 y 89%, mientras que con HDC se obtuvieron valores de entre el 77 y el 96%. Además, observaron que con AF4 no es posible la separación de NPs con su correspondiente forma iónica por posibles pérdidas de las especies iónicas en la membrana de separación, mientras que la cromatografía sí permite esta separación. Es decir, HDC-ICP-MS permite la obtención de información simultánea de NPs y especies

iónicas en un tiempo menor a 10 min. Este menor tiempo de análisis de HDC es otra ventaja adicional frente a AF4 [15]. Roman et al. [16] detectaron y cuantificaron simultáneamente Ag(I) y AgNPs en plasma y sangre mediante HDC acoplado SP-ICP-MS (HDC-SP-ICP-MS). El pico correspondiente a Ag(I) no se pudo separar correctamente de AgNPs de 20 nm, por lo que desarrollaron un algoritmo para deconvolucionar las señales de ambas especies y extrapolarlas a una caracterización con múltiples parámetros. Con una simple inyección, fueron capaces de obtener la concentración de Ag(I), la distribución de AgNPs en términos de su diámetro hidrodinámico y su concentración en masa y en número.

Todo esto hace que HDC-ICP-MS sea una buena candidata para ser una técnica complementaria para la especiación de nanopartículas y especies iónicas, debido a esta capacidad de separar ambas especies [17]. A pesar del potencial, no hay muchos estudios en los que se determinen de forma simultánea NPs y sus correspondientes formas iónicas con HDC. En un *review* publicado recientemente [17] se hace una revisión de los métodos publicados para la determinación y separación de AgNPs y sus correspondientes formas iónicas. Los dividen en métodos espectrométricos, métodos de separación y métodos electroanalíticos. En concreto, en los métodos para la separación de AgNPs y sus formas iónicas se exponen cuatro técnicas, la extracción en fase sólida, la extracción en punto de nube, AF4 y HDC. Elegir unas u otras depende de las necesidades del propio análisis y de las muestras a analizar. El tiempo de medida, la simplicidad de la técnica o la conservación de las propiedades de los analitos son parámetros a tener en cuenta en la elección de la técnica.

Las técnicas electroquímicas también permiten realizar esta especiación [17,18]. Mediante potenciometría con electrodos selectivos de iones es posible discriminar entre Ag(I) y otras especies de plata debido a que proporciona información sobre la actividad iónica y no sobre concentraciones totales [19]. Se ha conseguido detectar Ag(I) y AgNPs simultáneamente con la modificación de los electrodos de *Glassy-carbon* mediante electro-polimerización con poli(L-lisina) y usando voltamperometría, lo que permitió además conocer el tamaño de AgNPs [20]. Hernández et al. [21] desarrollaron un método voltamperometría de redisolución anódica de onda cuadrada para la determinación de

Ag(I) en presencia de AgNPs, consiguiendo límites de detección y cuantificación (LOQ) bajos (1.3 mg L^{-1} y 4.2 mg L^{-1} , respectivamente).

La cromatografía de exclusión por tamaño (SEC) es otra técnica de separación líquida utilizada en la bibliografía para la separación de NPs y metal disuelto [8,22–26]. Es una técnica similar a HDC y a AF4, debido a que su separación también se basa en el tamaño de las partículas y su orden de dilución es el mismo. Pero, a diferencia de HDC, la base de la separación en SEC se debe a que, durante el paso del analito por la columna, las partículas más grandes entran en un número menor de poros que las partículas más pequeñas [26], mientras que en HDC, la separación se debe al desarrollo de un perfil parabólico, que permite a las partículas más grandes ir en el centro de la parábola, evitando el contacto con las paredes [27]. Dong et al. [23] desarrollaron un método para la determinación simultánea de Ag(I) y plata particulada en muestras de hígado de rata con adiciones de Ag(I) y AgNPs, obteniendo buenos valores de recuperación para las partículas más pequeñas (10 y 20 nm) y para Ag(I), que se acercan al 100%, y buenos valores de resolución entre Ag(I) y AgNPs de 10, 20 y 30 nm (entre 1.05 y 2.37). Siguiendo este protocolo y optimizándolo, más recientemente, llevaron a cabo una especiación de plata en órganos (hígado, bazo, riñón y pulmón) de rata, siendo capaces de cuantificar y caracterizar AgNPs y Ag(I) con éxito [24]. Fernández-Trujillo et al. [28] optimizaron un método basado en HPLC, trabajando en modo de exclusión de tamaño, acoplado a un espectrómetro de masas de triple cuadrupolo de plasma acoplado inductivamente (ICP-TQ-MS), para la especiación de las diferentes formas de platino en diferentes medios de cultivo celular, consiguiendo separar el platino iónico de las nanopartículas de platino (PtNPs) de 5 nm en menos de 8 min. Obtuvieron LOD, del orden de $\mu\text{g L}^{-1}$, y buenas recuperaciones para NPs de 5 nm (100%), aunque para PtNPs de 30 nm, la recuperación disminuyó hasta el 66%. Betts et al. [29] utilizaron SEC acoplada a ICP-MS para la especiación de Ag(I) en contacto con medios bacterianos, siendo capaces de diferenciar los diferentes complejos de tamaños distintos que se forman cuando la plata se une a las diferentes proteínas. Fernández-Bautista et al. [30] también realizaron la especiación mediante SEC acoplado a ICP-MS y UV-Vis, pero, en este caso, de Se y Hg en músculo de diferentes especies de peces, siendo capaces de diferenciar también los diferentes tamaños de los complejos de los metales unidos a las moléculas. Aunque SEC es una técnica también eficiente y rápida, pueden darse interacciones entre

la fase estacionaria de la columna y el analito, debido a la porosidad del empaquetamiento [31,32]. En comparación con HDC, esta última presenta valores de dispersión más bajos [33].

El objetivo de este capítulo ha sido el desarrollo y evaluación de un método que permita la detección, cuantificación y caracterización de nanopartículas de oro y plata y sus correspondientes formas iónicas, mediante HDC-ICP-MS de forma sencilla. Para ello, se ha optimizado la fase móvil utilizada y distintos parámetros cromatográficos. Para cumplir este objetivo, se ha puesto especial interés en la resolución entre la especie iónica y las nanopartículas, y en la recuperación de las distintas especies en la columna. La validación del método propuesto se ha llevado a cabo mediante el análisis de suplementos nutricionales que contenían diferentes formas particuladas de oro y plata y sus especies iónicas correspondientes, comparando estos resultados con los obtenidos por TEM, FESEM y ultrafiltración con detección por espectroscopía de absorción atómica en llama (FAAS).

2. Materiales y métodos

2.1. Instrumentación

En este estudio se utilizó un módulo de bioseparaciones Waters 2796 (Waters Corporation, Milford, EE. UU.), como sistema cromatográfico de alta resolución, con una columna PL-PSDA tipo 1 (Agilent Technologies, Alemania), con un rango de separación nominal de 5 a 300 nm, una longitud de 80 cm y un diámetro interno de 7.5 mm. Esta columna se unió a un detector UV-Vis (detector de matriz de diodos, Waters 996), y se acopló a un ICP-MS (ELAN DCR-e, Perkin Elmer, Toronto, Canadá), que a la vez actuaba como detector. La salida de la columna se conectó directamente con el ICP-MS a través de un nebulizador de vidrio concéntrico *Slurry* con una cámara ciclónica (Glass Expansion, Melbourne, Australia). En la tabla 1.1. se muestran las condiciones experimentales utilizadas para el análisis por ICP-MS y por HDC.

Tabla 1.1. *Parámetros de medida para HDC-ICP-MS.*

ICP-MS	
Potencia RF (W)	1100
Flujo del gas de argón	
Plasma (L min ⁻¹)	15
Auxiliar (L min ⁻¹)	1.2
Nebulizador (L min ⁻¹)	1.0
Barridos por lectura	1
Tiempo de lectura (ms)	50
Modo de adquisición	Peak hopping
Isótopos monitorizados	¹⁰⁷ Ag, ¹⁰⁹ Ag, ¹⁹⁷ Au
HDC	
Columna	PL-PSA tipo 1
Caudal (mL min ⁻¹)	1.6
Volumen de inyección (μL)	50

Para la determinación de la concentración de plata total en los suplementos alimenticios, también se empleó un espectrómetro de absorción atómica (FAAS) (Perkin Elmer, Toronto, Canadá) modelo AAnalyst 200 con una llama de aire/acetileno. Se utilizó una lámpara de cátodo hueco de plata *Intensitron* (Perkin Elmer, Massachusetts, EE.UU.). La longitud de onda utilizada fue 328.1 nm y la anchura de rendija de 0.7 nm.

Para la determinación de la concentración de oro total en el suplemento alimenticio se utilizó un ICP-MS (ELAN DCR-e, Perkin Elmer, Toronto, Canadá) con un nebulizador de vidrio concéntrico *Slurry* con una cámara ciclónica (Glass Expansion, Melbourne, Australia). Las condiciones experimentales son las que se muestran en la tabla 1.2.

Tabla 1.2. *Parámetros de medida para ICP-MS.*

ICP-MS	
Potencia RF (W)	1100
Flujo del gas de argón	
Plasma (L min ⁻¹)	15
Auxiliar (L min ⁻¹)	1.2
Nebulizador (L min ⁻¹)	1.0
Barridos por lectura	50
Tiempo de lectura (ms)	50
Isótopos monitorizados	¹⁹⁷ Au

Para el estudio de los suplementos alimenticios de oro por TEM se utilizó un microscopio JEOL-2000 FXII (JEOL Ltd., Tokio, Japón) a 200 kV. Por otro lado, los suplementos alimenticios de plata fueron estudiados por FESEM con espectrometría de dispersión de energía (EDS). Estas observaciones se realizaron en un microscopio Merlin™ FESEM

equipado con una columna Gemini (Carl Zeiss Nano Technology Systems, Alemania), trabajando a 5 kV y con un detector de electrones secundarios *in-Lens*, con el fin de mejorar la resolución. El microscopio se combinó con un microanalizador de rayos X X-Max (Oxford Instruments, Reino Unido). En ambos casos, se depositaron 20 μL de muestra sobre una rejilla de cobre y el solvente se dejó secar a temperatura ambiente. En el caso de las medidas con FESEM, una vez seco, las muestras se cubrieron con carbono. Las imágenes obtenidas se procesaron con el software Image-J (Wayne Rasband et al., National Institutes of Health, EE.UU.) versión 1.53, con el que se determinó el promedio de los diámetros de partícula, midiendo aleatoriamente el tamaño de un número de partículas de las imágenes.

2.2. Reactivos

Las suspensiones de nanopartículas de oro y plata utilizadas se prepararon a partir de suspensiones comerciales (NanoComposix, San Diego, EE.UU.). Las suspensiones de AuNPs, estabilizadas en citrato 2 mM, tenían una concentración de 50 mg L^{-1} y diámetros nominales de 10, 50 y 100 nm (11.6 ± 1 , 52 ± 6 y 97 ± 11 nm, respectivamente). Las suspensiones de AgNPs utilizadas, estabilizadas también con citrato 2 mM, tenían una concentración de 21 mg L^{-1} y diámetros nominales de 10, 20, 40 y 60 nm (10.3 ± 2.1 , 18.6 ± 2.7 , 39 ± 5 , 59 ± 6 nm, respectivamente). También se utilizaron estándares de Au(III) ($1001 \pm 4 \text{ mg L}^{-1}$) y Ag(I) ($994 \pm 3 \text{ mg L}^{-1}$) (Fluka BioChemika, Buncchs, Suiza). Las disoluciones se prepararon por pesada con una balanza analítica (GH-200, A&D, California, EE. UU.).

Para la preparación de las distintas fases móviles estudiadas, se empleó hidrogenofosfato de sodio monohidratado (Na_2HPO_4), DL-penicilamina (PA) y Triton X-100 (Sigma-Aldrich, Stenheim, Alemania); dodecilsulfato de sodio (SDS) (Laboratorios Bio-Rad, Hercules, EE.UU.) y una disolución de formaldehído al 37 % (m/m) (Probus, Barcelona, España). El pH de todas las fases móviles fue de 7.5, que se ajustó con disoluciones de ácido nítrico (HNO_3) (69/70%, J.T.Baker, Phillipsburg, EE.UU.) e hidróxido de sodio (NaOH) (Prolabo, Barcelona, España), con un medidor de pH GLP 21 (Crison, España). También se utilizó ácido clorhídrico (HCl) (37%, J.T.Baker, Phillipsburg, EE.UU.) para la determinación de la concentración de oro total.

Tanto para la preparación de las fases móviles como para las diluciones se usó agua ultrapura (Milli-Q Advantage, Molsheim, Francia).

2.3. Muestras

Las muestras analizadas fueron suplementos nutricionales líquidos que contenían nanopartículas de oro y plata coloidal en su composición, adquiridos en las páginas web de distintos vendedores.

El suplemento nutricional de oro, denominado *Mesogold*, indicaba en su composición que contenía AuNPs con una concentración de 20 mg L^{-1} y un tamaño nominal de 3.2 nm.

Los suplementos de plata coloidal analizados fueron *Biovedik*, *High Stability* y *Wellness*. Se recomiendan como productos para la salud de administración oral o como desinfectantes de superficies para uso externo. *Biovedik* (en adelante suplemento 1) es un suplemento alimenticio con propiedades antibacterianas que posee plata coloidal con una concentración indicada de 25 mg L^{-1} . *High Stability* (en adelante suplemento 2) se vende como antibiótico, antiviral y antifúngico, compuesto por plata coloidal con una concentración indicada de 10 mg L^{-1} , y un tamaño de partícula de entre 0.6 y 5 nm. *Wellness* (en adelante suplemento 3) es una suspensión de plata coloidal con una concentración indicada de 30 mg L^{-1} y un rango de tamaño de AgNPs de entre 1 y 100 nm.

Todos los productos se almacenaron en ausencia de luz y a temperatura ambiente hasta su análisis.

2.4. Procedimientos

2.4.1. Determinación de la concentración total de oro y plata en los suplementos nutricionales

Para determinar la concentración total de oro, a $100 \text{ }\mu\text{L}$ de una suspensión de *Mesogold* se le añadieron $150 \text{ }\mu\text{L}$ de HNO_3 y $310 \text{ }\mu\text{L}$ de HCl [34]. Tras 1 hora a temperatura ambiente, las disoluciones resultantes se diluyeron en agua ultrapura hasta 10 mL. Las muestras se midieron por ICP-MS, utilizando una disolución de $10 \text{ }\mu\text{g L}^{-1}$ de Rh como

patrón interno y una recta de calibrado de concentraciones entre 0 y 50 $\mu\text{g L}^{-1}$ de Au(III) en 1 % de HNO_3 y 3% de HCl.

Para la determinación de la concentración total de los suplementos de plata, a 750 μL de la suspensión comercial se añadieron 750 μL de HNO_3 , dejándose 1 hora a temperatura ambiente. Posteriormente, las disoluciones se diluyeron con agua ultrapura hasta 5 mL y se midieron por FAAS, con una recta de calibrado de concentraciones entre 0 y 5 mg L^{-1} de Ag(I) en 1% de HNO_3 .

2.4.2. Determinación de la concentración de las especies iónicas de oro y plata en los suplementos nutricionales

Las especies iónicas de los suplementos nutricionales se aislaron, separándolas de las formas particuladas, mediante cartuchos de ultrafiltración *Nanosep Pall* con membranas de 3 kDa (tamaño equivalente a 2 nm de diámetro hidrodinámico). Inicialmente, los dispositivos se lavaron dos veces por centrifugación con 500 μL de agua ultrapura. A continuación, 500 μL de las disoluciones originales de los suplementos, previamente sonicadas, se centrifugaron a 14500 g durante 30 minutos a 20°C (en una centrífuga Thermo Heraeus XIR, equipada con un rotor de tubos *ependorf*, Waltham, EE. UU.). El ultrafiltrado resultante se diluyó a 10 mL con una disolución de HCl al 5% (v/v) para las disoluciones de oro y a 5 mL con HNO_3 al 1% (v/v) para las disoluciones de plata. Posteriormente, se midieron por FAAS siguiendo las condiciones instrumentales que se detallan en el apartado de instrumentación (sección 2.1). Se midió también una recta de calibrado con concentraciones entre 0 y 5 mg L^{-1} de Ag(I) en 1% de HNO_3 y Au(III) en 1.5 % de HNO_3 y 3% de HCl.

2.4.3. Determinación y cuantificación de distintas especies de oro y plata por HDC-ICP-MS

Con el objetivo de detectar y cuantificar las distintas especies de oro y plata (tanto iónico como en forma de nanopartículas) de los suplementos nutricionales, se optimizó la composición de la fase móvil. Para ello, se utilizaron patrones de AuNPs y Au(III) (250 $\mu\text{g L}^{-1}$), y AgNPs y Ag(I) (250 $\mu\text{g L}^{-1}$), que se separaron por HDC y se detectaron

posteriormente por ICP-MS, utilizando los parámetros instrumentales descritos en la tabla 1.1.

En la tabla 1.3. se muestra la composición de las distintas fases móviles empleadas. Antes de su uso, estas fases móviles se filtraron con filtros de nylon con un tamaño de 0.45 μm y se sonicaron.

Tabla 1.3. Composición de las fases móviles utilizadas en las separaciones realizadas por HDC-ICP-MS.

Separación de especies	Fases móviles
Oro	0.5 mM Na_2HPO_4 , 0.45 mM SDS, 0.05% Triton X-100, 0.05% formaldehído (Fabricante)
	0.45 mM SDS
	0.45 mM SDS, 0.05–0.5 mM PA
Plata	0.5 mM Na_2HPO_4 , 0.45 mM SDS, 0.05% Triton X-100, 0.05% formaldehído, 0.05–1mM PA (Fabricante)
	0.22 – 0.45 mM SDS, 1 mM PA

Antes de cada medida, se siguió el protocolo de acondicionamiento del cromatógrafo descrito por el fabricante: se realizaron las purgas correspondientes para la eliminación de burbujas del sistema, se lavó y homogeneizó la jeringuilla de inyección (volumen de 20 μL) y se acondicionó la columna con cada fase móvil, al menos durante 15 minutos.

Para la cuantificación de la concentración de oro y plata en las muestras, se preparó una recta de calibrado a partir del elemento disuelto, con concentraciones de 0 a 0.3 mg L^{-1} , que se midieron por ICP-MS utilizando el mismo sistema cromatográfico, pero sin columna, asegurándonos así de que no se produjeran interacciones y retenciones en la columna. En la recta de calibrado, el área de pico obtenida en cada caso se representó frente a la concentración del metal iónico. Para la cuantificación de cada muestra, se midió el área de los picos obtenidos, cuyo valor se interpoló en la recta de calibrado previamente elaborada.

Para la representación y el procesamiento de los datos adquiridos se utilizó el *software* Origin 9.6.5.169 (OriginLab, Northampton, MA, EE. UU.).

2.4.3.1. Cálculo de recuperación de columna

Las posibles pérdidas de analito en el sistema cromatográfico por interacción de éste con la fase estacionaria se determinaron mediante el cálculo de la recuperación de columna, que se obtiene con la relación del área de los picos obtenidos medidos con y sin columna (ecuación (1.1)), para los estándares de NPs y especies iónicas con los mismos parámetros instrumentales [10]. Para el cálculo de la recuperación se utilizaron disoluciones de los estándares de Au(III) y Ag(I) en penicilamina y de AuNPs (10, 50 y 100 nm) y AgNPs (10, 20, 40 y 60 nm) en agua ultrapura, con una concentración de 250 µg L⁻¹.

$$\% \text{Recuperación} = \frac{\text{Área de pico con columna}}{\text{Área de pico sin columna}} \cdot 100 \quad (1.1)$$

2.4.3.2. Cálculo de la resolución

La resolución es un parámetro cromatográfico que indica la capacidad de la técnica para separar los picos de dos especies distintas. Se calculó mediante la ecuación (1.2) donde $t_{r,1}$ y $t_{r,2}$ son los tiempos de retención, y w_1 y w_2 son las anchuras de los picos, de nanopartículas y de la especie iónica, respectivamente.

$$R = \frac{2(t_{r,2} - t_{r,1})}{w_1 + w_2} \quad (1.2)$$

3. Resultados y discusión

3.1. Optimización de la fase móvil

En las técnicas basadas en cromatografía líquida, la composición de la fase móvil es un parámetro clave para conseguir mejores separaciones. La fuerza iónica, las interacciones del analito con la fase estacionaria y con la fase móvil, así como el pH de ésta, son algunos de los parámetros que influyen, tanto en el tiempo de retención y, por lo tanto, en la resolución, como en la recuperación de columna.

En la mayoría de los trabajos publicados en los que se ha utilizado HDC, el objetivo es la separación de nanopartículas de distintos tamaños. Sin embargo, en el presente trabajo el principal objetivo es desarrollar un método cromatográfico que permita la separación de

las formas iónicas de oro y plata de sus correspondientes formas nanoparticuladas, con valores de recuperación y resolución adecuados. Con este objetivo, se ha optimizado la composición de las distintas fases móviles, que se muestran en la tabla 1.3 del apartado de procedimientos (sección 2.4.3), y se han estudiado sus parámetros cromatográficos para obtener los mejores valores de recuperación y resolución posibles.

La composición de la fase móvil recomendada por el fabricante de la columna de HDC (Fabricante) para la separación de NPs es: 0.5 mM de Na_2HPO_4 , 0.45 mM de SDS, 0.05% de Triton X-100 y 0.05% de formaldehído, con un pH de 7.5. Triton X-100 es un surfactante no-iónico que estabiliza las nanopartículas, cubriendo su superficie y evitando, de esa forma, su agregación [35], mientras que SDS es un surfactante aniónico que previene la adsorción de las partículas en la columna [10,28,36]. El fosfato se utiliza para mantener el pH y la fuerza iónica de la fase móvil [34] y el formaldehído como bactericida. Esta fase móvil es la más utilizada en la bibliografía, como tal o con pequeñas modificaciones [10,16,34,37]. El uso de fases móviles con baja fuerza iónica provoca que las partículas se repelan de la superficie de la fase estacionaria y, en consecuencia, la separación y la resolución mejore [34,38].

En el caso del oro, primero se evaluó la fase móvil recomendada por el fabricante, utilizando un caudal de 1.6 mL min^{-1} . Los cromatogramas obtenidos para estándares de AuNPs de 10, 50 y 100 nm y para Au(III) (con una concentración de $250 \mu\text{g L}^{-1}$) se muestran en la figura 1.1a. Los tiempos de retención fueron 8.26, 8.08 y 7.94 min, para 10, 50 y 100 nm, respectivamente (tabla 1.4). Las recuperaciones obtenidas estaban en el rango de 68 y 77% (tabla 1.5). Sin embargo, no se observó la aparición del pico correspondiente a Au(III) debido a que esta especie se retenía completamente en la columna. Utilizando una fase móvil más simple, cuya composición es 0.45 mM de SDS con un pH 7.5, los tiempos de retención de AuNPs disminuyeron hasta 8.10, 7.90 y 7.82 min para AuNPs de 10, 50 y 100 nm (figura 1.1b) (tabla 1.4). Aunque se obtuvieron mejores recuperaciones para AuNPs de 10 y 50 nm (88 y 83%, respectivamente) (tabla 1.5), la recuperación de AuNPs de 100 nm disminuyó hasta un valor de 44% y, además, igual que en el caso de la fase móvil anterior, no se observó el pico correspondiente a Au(III). La inestabilidad de Au(III) provoca una retención irreversible en la columna. Para evitarlo, se estabilizó el Au(III) mediante la adición de especies que formen ligandos de azufre con el oro, como penicilamina [39] o L-cisteína [40]. Solo con penicilamina se

obtenían valores reproducibles, por lo que se estudió su adición con distintas concentraciones (0.05, 0.2 y 0.5 mM). Las recuperaciones obtenidas se muestran en la tabla 1.5. Mientras que, la recuperación de AuNPs de 10 nm con la adición de penicilamina no se vio afectada, la recuperación de AuNPs de 50 nm y de 100 nm disminuyó a medida que aumentaba la concentración de penicilamina añadida a la fase móvil. El tiempo de retención de Au(III) (figura 1.1.c) (tabla 1.4), con una concentración de 0.05 mM de penicilamina, fue de 8.75 min y su recuperación fue del $94 \pm 3\%$. Por ello, debido a la baja recuperación obtenida para las AuNPs de diámetro más grande al aumentar la concentración de penicilamina, en los siguientes experimentos con oro se utilizó la fase móvil cuya concentración de penicilamina era de 0.05 mM.

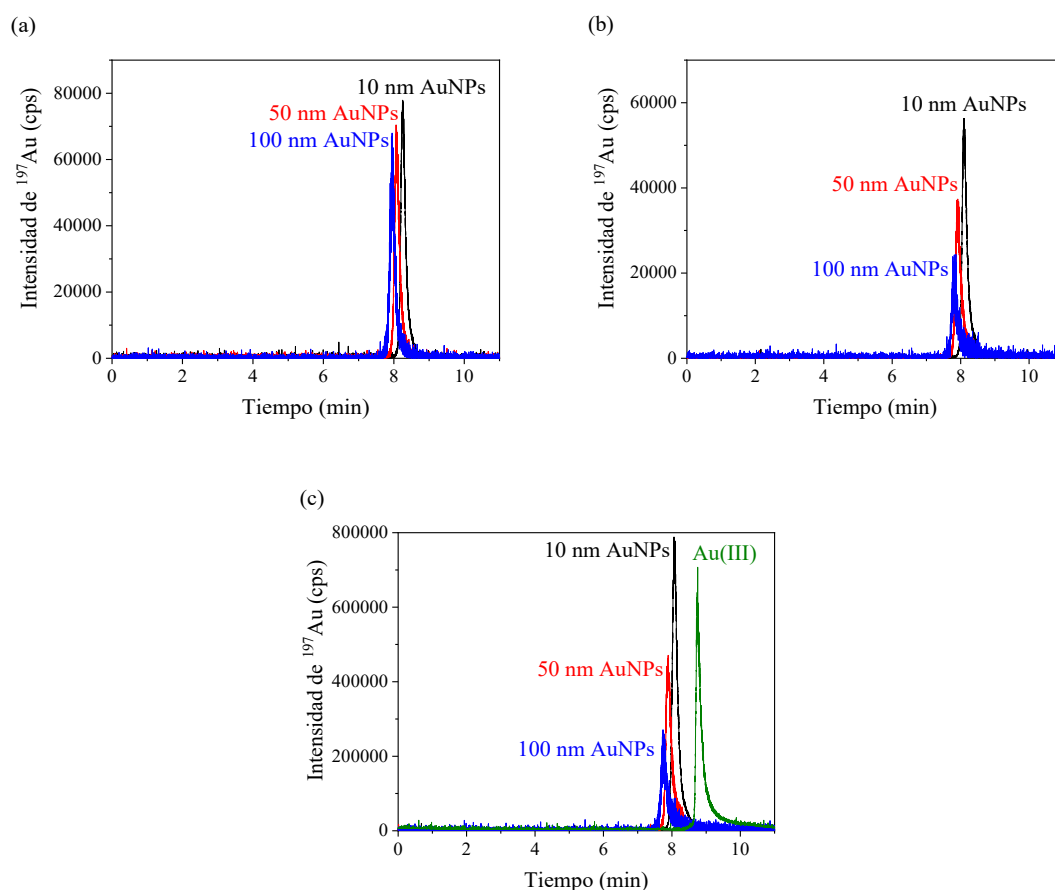


Figura 1.1. Cromatogramas obtenidos para AuNPs de 10, 50 y 100 nm y Au(III) usando diferentes fases móviles. (a) Fabricante (b) 0.45 mM SDS (c) 0.45 mM SDS y 0.05 mM de penicilamina.

Tabla 1.4. Tiempos de retención para Au(III) y AuNPs con las distintas fases móviles estudiadas. Media $\pm$ desviación estándar ($n=3$).

Fase móvil	Tiempos de retención (min)			
	Au(III)	AuNPs 10 nm	AuNPs 50 nm	AuNPs 100 nm
Fabricante	-	8.26 $\pm$ 0.01	8.08 $\pm$ 0.01	7.94 $\pm$ 0.01
0.45 mM SDS	-	8.10 $\pm$ 0.01	7.90 $\pm$ 0.01	7.82 $\pm$ 0.01
0.45 mM SDS+				
0.05 mM PA	8.76 $\pm$ 0.02	8.07 $\pm$ 0.01	7.89 $\pm$ 0.01	7.74 $\pm$ 0.01
0.20 mM PA	8.81 $\pm$ 0.03	8.01 $\pm$ 0.01	7.88 $\pm$ 0.07	7.74 $\pm$ 0.09
0.50 mM PA	8.77 $\pm$ 0.01	8.09 $\pm$ 0.01	7.92 $\pm$ 0.01	7.77 $\pm$ 0.02

Tabla 1.5. Recuperaciones obtenidas para Au(III) y AuNPs con las diferentes fases móviles estudiadas. Media $\pm$ desviación estándar ($n=3$).

Fase móvil	Recuperación de columna (%)			
	Au(III)	AuNPs 10 nm	AuNPs 50 nm	AuNPs 100 nm
Fabricante	-	77 $\pm$ 1	74 $\pm$ 1	69 $\pm$ 3
0.45 mM SDS	-	88 $\pm$ 1	83 $\pm$ 5	44 $\pm$ 2
0.45 mM SDS +				
0.05 mM PA	94 $\pm$ 3	103 $\pm$ 1	102 $\pm$ 2	72 $\pm$ 9
0.20 mM PA	88 $\pm$ 5	114 $\pm$ 2	82 $\pm$ 2	66 $\pm$ 4
0.50 mM PA	89 $\pm$ 6	116 $\pm$ 2	76 $\pm$ 8	61 $\pm$ 2

La resolución obtenida entre AuNPs de 10 nm y Au(III) fue de 0.70, un valor aceptable. No obstante, se estudió si este valor se podía mejorar trabajando a caudales más bajos, como 1.0 mL min⁻¹. El cromatograma utilizando este caudal se muestra en la figura 1.2. La resolución obtenida fue 1.03. Sin embargo, el aumento del tiempo de medida, que pasa de 11 min a casi 17 min, no compensa este aumento en la resolución, por lo que se decidió trabajar a un caudal de 1.6 mL min⁻¹.

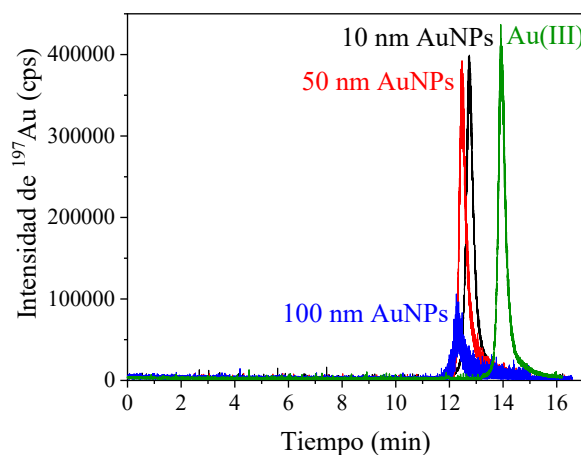


Figura 1.2. Cromatogramas obtenidos para AuNPs y Au(III) con fase móvil 0.45 mM SDS y 0.05 mM de penicilamina y con caudal de 1.0 mL min⁻¹.

En el caso de la plata, se procedió de la misma forma que con el oro, estudiando diferentes fases móviles. Inicialmente, se utilizó la recomendada por el fabricante añadiéndole distintas concentraciones de penicilamina para la estabilización de Ag(I). Los resultados de las recuperaciones obtenidas se muestran en la tabla 1.6 y los tiempos de retención en la tabla 1.7. Los mejores resultados se obtuvieron con una concentración de penicilamina de 1.0 mM (106% para Ag(I), y 89, 90 y 47% para AgNPs de 10, 40 y 60 nm, respectivamente). A pesar de estos valores de recuperación, la resolución que se obtuvo entre Ag(I) y AgNPs de 10 nm fue muy baja, de 0.16. Disminuyendo el caudal a 1.0 mL min⁻¹, como se había estudiado para el oro, la resolución no mejoró, obteniéndose un valor de 0.17, lo que coincide con la teoría de HDC, en la que se dice que el caudal no debería afectar a la eficiencia de separación [34].

Tabla 1.6. Recuperaciones obtenidas para Ag(I) y AgNPs con las diferentes fases móviles estudiadas. Media $\pm$ desviación estándar ($n=3$).

Fase móvil	Recuperación de columna (%)				
	Ag(I)	AgNPs 10 nm	AgNPs 20 nm	AgNPs 40 nm	AgNPs 60 nm
Fabricante +					
0.05 mM PA	25 $\pm$ 4	66 $\pm$ 5	66 $\pm$ 4	66 $\pm$ 9	24 $\pm$ 1
0.2 mM PA	40 $\pm$ 1	100 $\pm$ 5	107 $\pm$ 11	90 $\pm$ 9	49 $\pm$ 3
0.5 mM PA	35 $\pm$ 7	77 $\pm$ 6	81 $\pm$ 1	80 $\pm$ 9	76 $\pm$ 2
1.0 mM PA	106 $\pm$ 6	89 $\pm$ 6	-	90 $\pm$ 9	47 $\pm$ 1
1 mM PA +					
0.45 mM SDS	105 $\pm$ 20	89 $\pm$ 9	64 $\pm$ 6	83 $\pm$ 5	56 $\pm$ 6
0.34 mM SDS	103 $\pm$ 10	102 $\pm$ 3	72 $\pm$ 11	30 $\pm$ 6	8 $\pm$ 1
0.22 mM SDS	101 $\pm$ 10	90 $\pm$ 9	21 $\pm$ 4	10 $\pm$ 5	6 $\pm$ 1

Tabla 1.7. Tiempos de retención para Ag(I) y AgNPs con las distintas fases móviles estudiadas. Media $\pm$ desviación estándar ($n=3$).

Fase móvil	Tiempos de retención (min)				
	Ag(I)	AgNPs 10 nm	AgNPs 20 nm	AgNPs 40 nm	AgNPs 60 nm
Fabricante +					
0.05 mM PA	8.53 $\pm$ 0.01	8.26 $\pm$ 0.01	8.19 $\pm$ 0.01	8.08 $\pm$ 0.01	8.02 $\pm$ 0.02
0.20 mM PA	8.60 $\pm$ 0.01	8.27 $\pm$ 0.02	8.19 $\pm$ 0.01	8.10 $\pm$ 0.01	8.05 $\pm$ 0.02
0.50 mM PA	8.60 $\pm$ 0.01	8.25 $\pm$ 0.01	8.19 $\pm$ 0.01	8.07 $\pm$ 0.01	8.01 $\pm$ 0.02
1 mM PA	8.54 $\pm$ 0.02	8.35 $\pm$ 0.02	-	8.17 $\pm$ 0.02	8.12 $\pm$ 0.03
1 mM PA +					
0.45 mM SDS	8.65 $\pm$ 0.05	8.29 $\pm$ 0.01	-	8.10 $\pm$ 0.01	8.05 $\pm$ 0.01
0.34 mM SDS	8.63 $\pm$ 0.01	7.96 $\pm$ 0.03	7.91 $\pm$ 0.01	7.79 $\pm$ 0.03	7.73 $\pm$ 0.02
0.22 mM SDS	8.64 $\pm$ 0.01	7.80 $\pm$ 0.03	7.79 $\pm$ 0.02	7.65 $\pm$ 0.03	7.61 $\pm$ 0.01

La disminución de la fuerza iónica también provoca una reducción de los tiempos de retención de plata. Con una fuerza iónica alta, las interacciones de *Van der Waals* dominan sobre el efecto hidrodinámico, provocando una disminución en los tiempos de retención de AgNPs [41]. Una disminución en la concentración de SDS provoca una disminución de la fuerza iónica. Se estudiaron fases móviles con una concentración 1 mM de penicilamina (por ser la que mejores porcentajes de recuperación proporcionaba) y diferentes concentraciones de SDS sin añadir fosfatos. Se ha observado que la ausencia de fosfatos en la fase móvil lleva a mejores separaciones entre partículas grandes y las especies iónicas [34].

Los valores de resolución obtenidos con 1 mM penicilamina y las distintas concentraciones de SDS, se muestran en la tabla 1.8. Se puede observar que la resolución aumentaba al disminuir la concentración de SDS, llegando a obtener valores de 0.93, en el caso de la fase móvil con una composición de 0.22 mM de SDS y 1 mM de penicilamina. Como se ha señalado anteriormente, esto se debe a la disminución de la fuerza iónica, que provoca que los tiempos de retención de AgNPs disminuyan [41]. Sin embargo, los valores de recuperación obtenidos (tabla 1.6) fueron muy bajos, únicamente Ag(I) y AgNPs de 10 nm tiene valores cercanos al 100%. El valor de recuperación de AgNPs de 20, 40 y 60 nm no llega al 20%. Esto se debe a la inestabilidad y adsorción de las partículas más grandes en ella. Buscando un equilibrio entre los valores de resolución y recuperación se decidió seleccionar como fase móvil óptima, aquella con una concentración de SDS de 0.34 mM, en la que la resolución es de 0.63 y los valores de recuperación son aceptables para AgNPs de 20 nm, y del 100% para Ag(I) y AgNPs de 10 nm. En la figura 1.3 se muestran los cromatogramas obtenidos para los estándares de plata con las fases móviles con composición de 1 mM de penicilamina y distintas concentraciones de SDS.

Tabla 1.8. Resoluciones obtenidas para Ag(I) y AgNPs de 10 nm con las diferentes fases móviles estudiadas. Media $\pm$ desviación estándar ($n=3$).

Fase móvil	Resolución
1 mM PA +	
0.45 mM SDS	0.307 ± 0.015
0.34 mM SDS	0.634 ± 0.022
0.22 mM SDS	0.926 ± 0.082

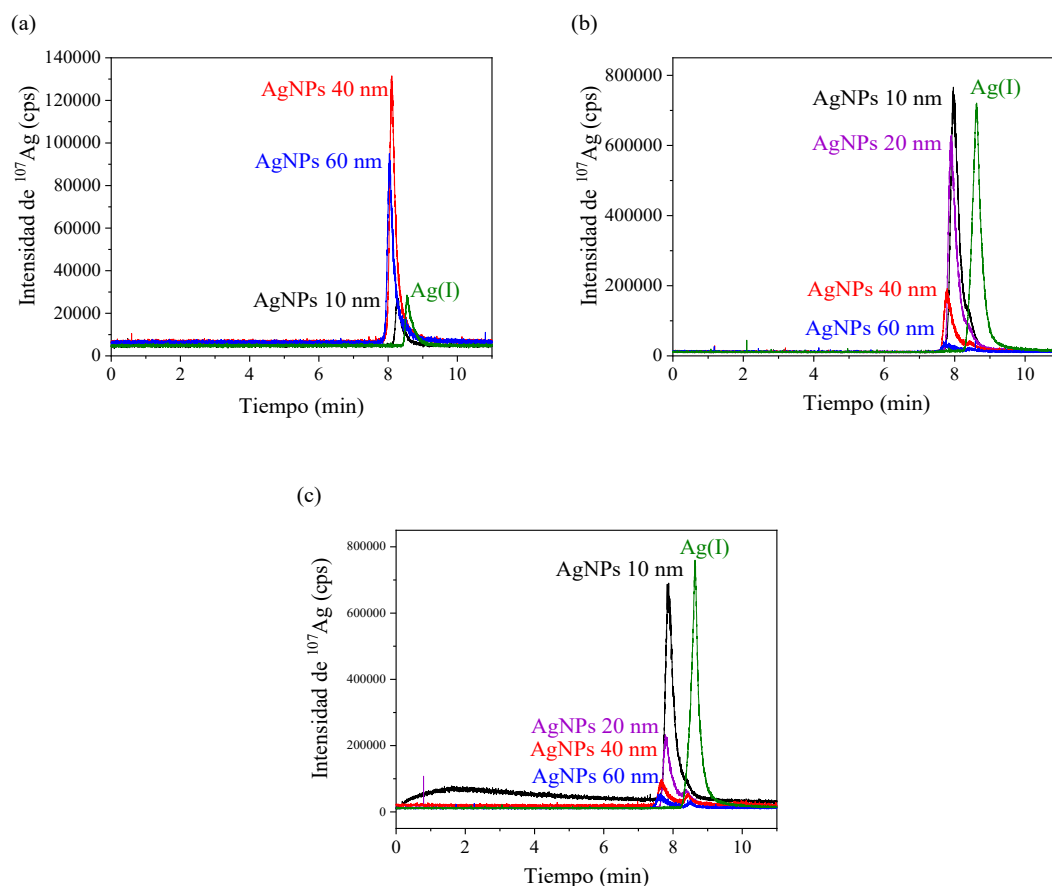


Figura 1.3. Cromatogramas obtenidos para AgNPs y Ag(I) usando diferentes composiciones de SDS y 1 mM de penicilamina. (a) 0.45 mM SDS (b) 0.34 mM SDS (c) 0.22 mM SDS.

3.2. Prestaciones analíticas

Se procedió al cálculo de los parámetros analíticos correspondientes: curvas de calibrado, tanto de concentración como de tamaño; linealidad, repetibilidad y LOD y LOQ.

En la bibliografía, para obtener el diámetro de partícula, se han utilizado dos formas de ajuste de curva de calibrado. Por un lado, un ajuste lineal [34,42], en el que se representan los tiempos de retención de nanopartículas de distinto tamaño frente a la raíz cuadrada del diámetro; y, por otro, un ajuste logarítmico [16,43–45], en el que se representa directamente el tiempo de retención de nanopartículas de distinto tamaño frente al diámetro. Para la obtención de las rectas de calibrado de tamaño se midieron patrones de AuNPs, con tamaños de 10, 50 y 100 nm, y patrones de AgNPs de 10, 20, 40 y 60 nm. Las rectas obtenidas para el oro y la plata utilizando la fase móvil escogida en cada caso,

se muestran en la figura 1.4a para el oro y figura 1.4b para la plata. Para comparar ambas rectas, se aplicó un test t a los tamaños obtenidos mediante ambos tipos de calibración, sin encontrar diferencias significativas entre ambos modelos de recta.

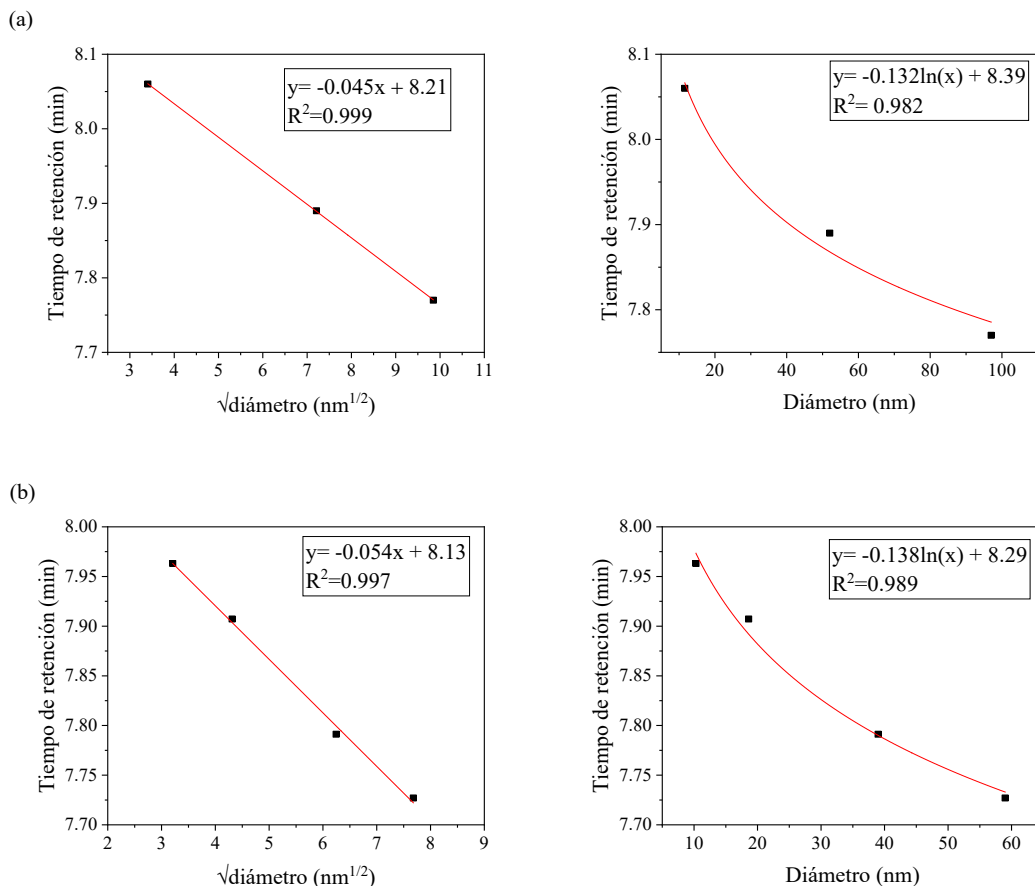


Figura 1.4. Curvas de calibrado (tamaño) (a) AuNPs con la fase móvil de composición 0.45 mM de SDS y 0.05 mM de penicilamina. (b) AgNPs con la fase móvil de 0.34 mM de SDS y 1 mM de penicilamina.

Como se ha indicado en procedimientos (sección 2.4.3), para poder llevar a cabo la cuantificación de las distintas especies de oro y plata en las muestras, se procedió a realizar una curva de calibrado midiendo el área obtenida para patrones de Au(III) y Ag(I) de concentración creciente sin columna, evitando así posibles interacciones y retenciones con la columna. Se representó el área de los picos resultantes frente a su concentración en masa y se ajustó a una recta. Las ecuaciones obtenidas fueron $y = 1.56 \cdot 10^4 x + 9.99 \cdot 10^4$ ($R = 0.9998$) para el oro y $y = 8.86 \cdot 10^5 x + 2.27 \cdot 10^3$ ($R = 0.9996$) para la plata.

Los LOD y LOQ en concentración se calcularon como la desviación estándar de la línea base, multiplicada por 3 y 10, respectivamente, dividida por la pendiente de la recta de

calibrado de la concentración en masa. Bajo estas condiciones, los límites de detección y cuantificación para oro encontrados fueron de 0.05 y 0.016 $\mu\text{g L}^{-1}$ y para plata fueron 0.75 y 2.5 $\mu\text{g L}^{-1}$.

3.3. Análisis de especies de oro y plata en suplementos nutricionales

Optimizados los parámetros cromatográficos, se procedió con el análisis de suplementos nutricionales.

Se determinó el contenido total de oro en el suplemento *Mesogold* por ICP-MS, siguiendo el procedimiento descrito en el apartado de métodos (sección 2.4.1), obteniéndose una concentración total de $21.0 \pm 0.8 \text{ mg L}^{-1}$, equivalente al valor proporcionado por el vendedor. Además, el suplemento se analizó por HDC-ICP-MS. El cromatograma obtenido con una dilución de 1:150 en agua ultrapura se muestra en la figura 1.5. En el cromatograma se observa la aparición de un pico a 7.99 min, que corresponde a nanopartículas con un tamaño de $6.5 \pm 0.01 \text{ nm}$, y un pequeño hombro a 8.47 min, que correspondería a Au(III). Este pequeño hombro no se separa bien del pico de las nanopartículas, debido al pequeño tamaño de estas y la baja concentración de Au(III) en la muestra, por lo que no se pudo cuantificar. La concentración obtenida por HDC-ICP-MS para AuNPs fue de $20.4 \pm 0.8 \text{ mg L}^{-1}$, lo que concuerda con el resultado obtenido por ICP-MS ($21.0 \pm 0.8 \text{ mg L}^{-1}$) y con el vendedor (20 mg L^{-1}).

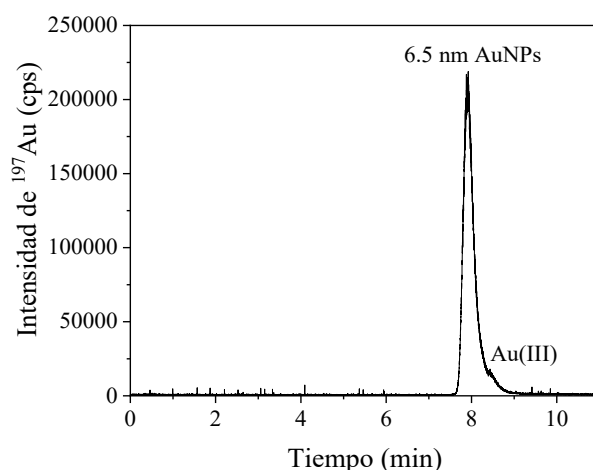


Figura 1.5. Cromatograma obtenido para el suplemento nutricional de oro *Mesogold* con fase móvil de 0.45 mM SDS y 0.05 mM penicilamina.

Este suplemento también se analizó por TEM, cuya imagen y distribución de tamaño se muestran en la figura 1.6. Esta distribución se obtuvo midiendo el diámetro de 87 partículas aleatorias con el *software* Image-J. El tamaño medio de esta distribución fue de 5.1 ± 2.4 nm, similar a lo encontrado por HDC-ICP-MS, aunque se observó la aparición de partículas más grandes, que llegan a los 10-12 nm.

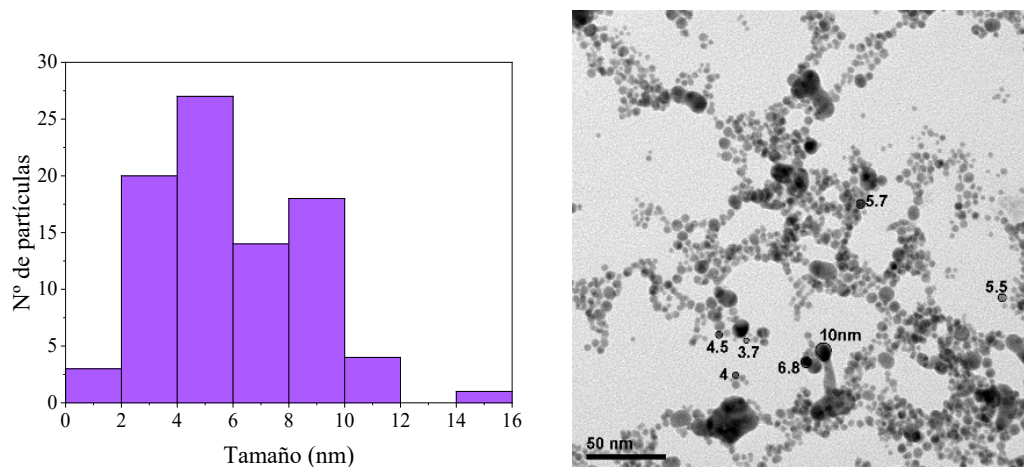


Figura 1.6. Imagen obtenida por TEM del suplemento nutricional de oro y su correspondiente distribución de tamaño.

Además, se realizó un estudio de recuperación adicionando una concentración de $155 \mu\text{g L}^{-1}$ de Au(III) sobre la muestra de suplemento que posteriormente se analizó por HDC-ICP-MS (figura 1.7). Se obtuvo una recuperación de Au(III) de $88.7 \pm 4.5\%$. El pico que corresponde a Au(III) se eluye a 8.78 min, y, en este caso, al aumentar la concentración sí se separó correctamente del pico de las nanopartículas.

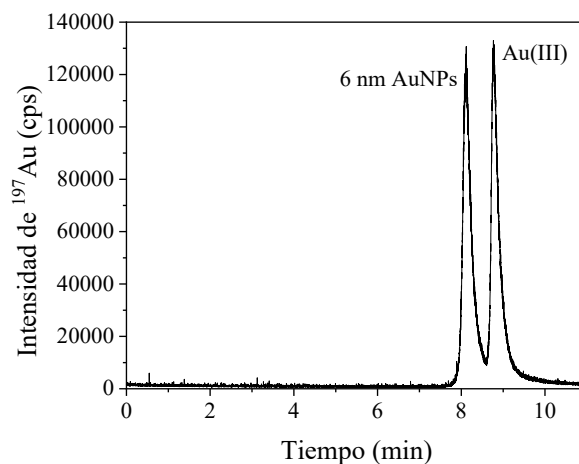


Figura 1.7. Cromatograma obtenido para el suplemento nutricional Mesogold con una adición de Au(III). Fase móvil: 0.45 mM SDS y 0.05 mM penicilamina.

De igual forma, se analizaron tres suplementos nutricionales que contenían plata coloidal y/o iónica, cuya composición se encuentra en el apartado de muestras (sección 2.3). Para cuantificar la plata total presente en las muestras, se analizaron por FAAS. Además, se llevó a cabo una ultrafiltración, utilizando cartuchos de membrana de 3 kDa, de las muestras para cuantificar la plata iónica. Los resultados y la comparativa con las concentraciones obtenidas por HDC-ICP-MS se muestran en la tabla 1.9. La recuperación de Ag(I) con los dispositivos de ultrafiltración fue de $92.8 \pm 0.3\%$. Los tres suplementos fueron analizados utilizando como fase móvil aquella cuya composición era de 0.34 mM de SDS y 1 mM de penicilamina, por ser la que mantenía el equilibrio entre recuperaciones de AgNPs y resolución. Los valores de plata total obtenidos por HDC-ICP-MS fueron similares a los obtenidos por FAAS. Además, estas muestras se cuantificaron en otro estudio mediante voltametría de resolución anódica [21], y se obtuvieron resultados similares.

Tabla 1.9. Concentraciones y tamaños obtenidas para los suplementos nutricionales de plata. Media $\pm$ desviación estándar ($n=3$).

Muestra	HDC-ICP-MS			FAAS	
	AgNPs (mg L ⁻¹)	Ag(I) (mg L ⁻¹)	Plata total (mg L ⁻¹)	UF-FAAS Ag(I) (mg L ⁻¹)	Plata total (mg L ⁻¹)
Suplemento 1	4.30 ± 1.20	14.2 ± 0.5	18.5 ± 2.4	16.8 ± 0.5	21.3 ± 2.1
Suplemento 2	*	10.9 ± 0.6	10.9 ± 0.6	11.6 ± 1.0	12.0 ± 1.6
Suplemento 3	26.3 ± 0.3	*	26.3 ± 0.3	1.70 ± 0.60	37.5 ± 0.8

* No cuantificado por solapamiento de picos

Los cromatogramas de los suplementos se muestran en la figura 1.8. Para el suplemento 1 (figura 1.8a) se obtuvo un pico a 8.48 min, que corresponde a Ag(I) y un pico más pequeño a 7.99 min que corresponde a AgNPs con un tamaño de 23.7 ± 0.8 nm. La resolución entre ambos picos fue buena, lo que permitió que su cuantificación pudiese realizarse sin problemas. En el caso del suplemento 2 (figura 1.8b), se obtuvo un pico a 8.46 min y un hombro a su izquierda a 8.10 min que corresponde a partículas de 11.6 ± 2.2 nm; y en el caso del suplemento 3 (figura 1.8c), se observaron dos picos a 7.89 min y 8.48 min, que corresponden a AgNPs de 47.9 ± 0.03 nm y Ag(I), respectivamente. En el caso de estos dos últimos suplementos, la resolución fue muy baja y los picos se solapaban parcialmente. Esto provocó que la cuantificación de cada uno de los picos por separado

no fuera del todo correcta en el caso del suplemento 3 y que, para el suplemento 2, no se pudiese llevar a cabo.

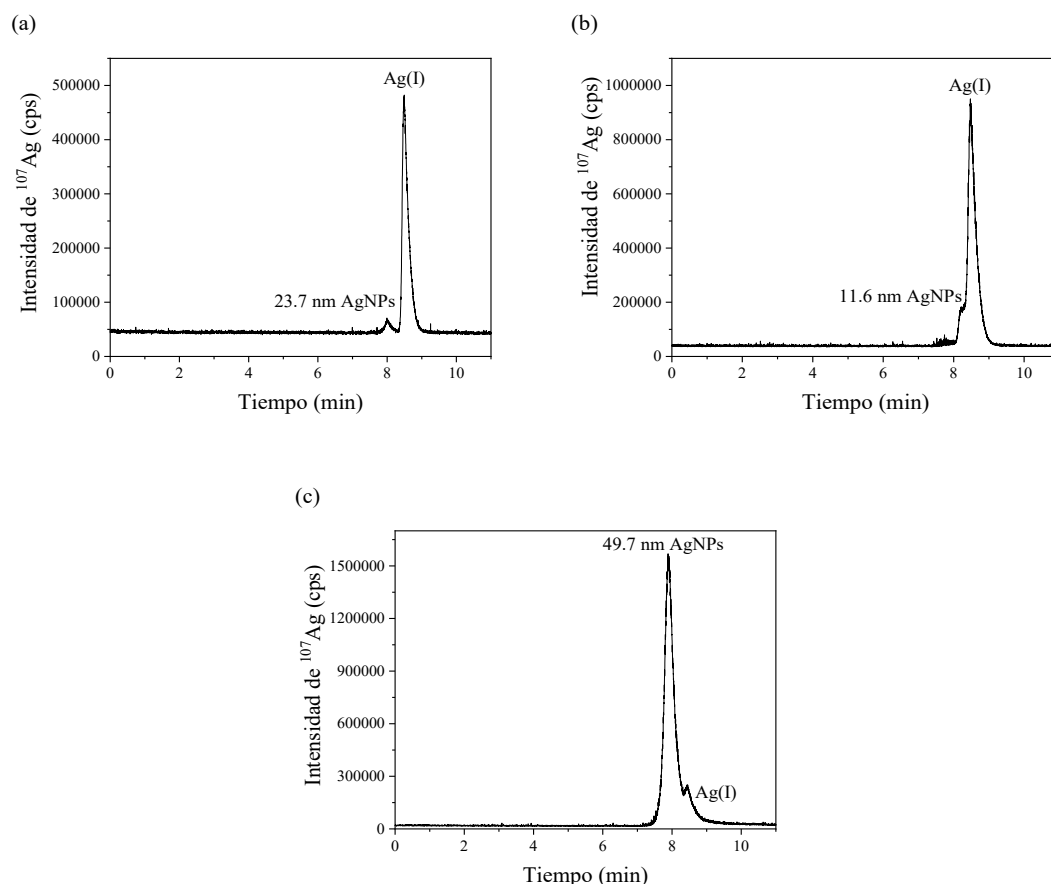


Figura 1.8. Cromatogramas obtenidos para los suplementos nutricionales que contienen plata. (a) Suplemento 1 (b) Suplemento 2 (c) Suplemento 3. Fase móvil: 0.34 mM SDS y 1 mM penicilamina.

Los suplementos de plata fueron también analizados por FESEM. La figura 1.9 muestra las imágenes obtenidas para los suplementos 1 y 2 y sus correspondientes distribuciones de tamaño calculadas utilizando el *software* Image-J. En el caso de las imágenes del suplemento 3, no se observaron de forma clara nanopartículas, debido probablemente a su composición (alginato potásico y EDTA). Esto hizo que no fuera posible el análisis posterior de las partículas. Para el suplemento 1 (figura 1.9a), el tamaño medio encontrado fue de 23.4 ± 5.8 nm y para el suplemento 2 (figura 1.9b), 14.2 ± 6.8 nm. Estos tamaños coinciden con los tamaños obtenidos por HDC-ICP-MS (23.7 ± 0.8 nm y 11.6 ± 2.2 nm para los suplementos 1 y 2, respectivamente).

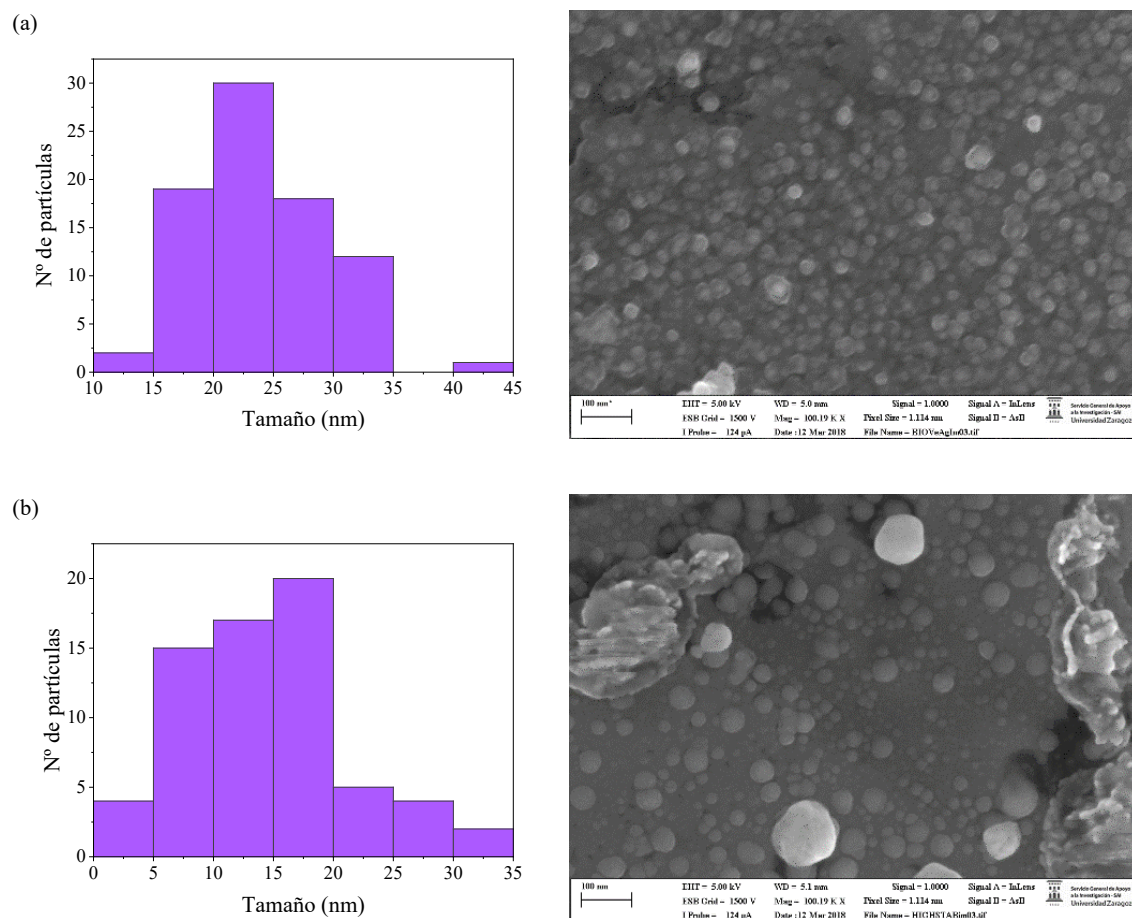


Figura 1.9. Distribuciones de tamaño y sus correspondientes imágenes obtenidas por FESEM de los suplementos nutricionales. (a) Suplemento 1 (b) Suplemento 2.

3.3.1. Comparativa de los resultados obtenidos

El método optimizado basado en HDC-ICP-MS para la especiación de especies particuladas e iónicas de oro y plata permite la caracterización y cuantificación de las especies iónicas y de las nanopartículas, siempre que estas últimas sean lo suficientemente grandes y la concentración de las especies sea lo suficientemente alta para que los picos de las especies puedan ser resueltos de manera correcta.

En la tabla 1.10 se muestran todos los resultados de tamaño y concentración obtenidos por las distintas técnicas utilizadas para los suplementos nutricionales, así como los valores teóricos dados por los vendedores. En cuanto al suplemento de oro (*Mesogold*), los resultados, tanto de concentración, como de tamaño, se ajustaron bastante bien a los valores teóricos.

En el caso de los suplementos de plata, en general, los valores obtenidos por los distintos métodos también fueron comparables. Para el suplemento 2, la concentración que se obtuvo por HDC-ICP-MS ($10.9 \pm 0.6 \text{ mg L}^{-1}$) encajó con el valor indicado (10 mg L^{-1}), mientras que en el caso de los suplementos 1 ($18.5 \pm 2.4 \text{ mg L}^{-1}$) y 3 ($26.3 \pm 0.3 \text{ mg L}^{-1}$), la concentración fue algo más baja (concentraciones indicadas para los suplementos 1 y 3 de 25 y 30 mg L^{-1} , respectivamente). Para el suplemento 1, la concentración obtenida por FAAS ($21.3 \pm 2.1 \text{ mg L}^{-1}$) coincidió con la obtenida por HDC, por lo que se puede concluir que la concentración obtenida fue correcta. En el caso del suplemento 3, la imposibilidad de resolver de forma correcta el pico correspondiente a Ag(I), debido al solapamiento de éste con el pico de AgNPs, hizo que la concentración obtenida fuera menor. En el caso de los tamaños obtenidos, para el suplemento 1 el vendedor no da información sobre el tamaño teórico de las nanopartículas añadidas, no obstante, los tamaños obtenidos por HDC-ICP-MS (23.7 nm) y TEM (23.4 nm) fueron similares. En el caso del suplemento 3, el tamaño obtenido por HDC-ICP-MS (49.7 nm) estaba en el rango dado por el vendedor (1-100 nm). Sin embargo, en el caso del suplemento 2, el tamaño que se obtuvo por HDC-ICP-MS (11.6 nm) fue superior al dado por el vendedor (0.6-5 nm). Si bien, por TEM, el tamaño medio obtenido fue similar a éste (14.2 nm), con lo que se puede concluir que el tamaño que se obtuvo por HDC era correcto.

Tabla 1.10. Concentraciones y tamaño obtenidos para los diferentes suplementos alimenticios de oro y plata. Media $\pm$ desviación estándar ($n=3$).

Concen.	Teórico	HDC-ICP-MS			UF-ICP-MS	ICP-MS	TEM
	Oro total (mg L⁻¹)	AuNPs (mg L⁻¹)	Au(III) (mg L⁻¹)	Oro total (mg L⁻¹)	Au(III) (μg L⁻¹)	Oro total (mg L⁻¹)	
<i>Mesogold</i>	20	20.4 $\pm$ 0.8	*	20.4 $\pm$ 0.8	<2.3 (LOQ)	21.0 $\pm$ 0.8	
Tamaño	AuNPs (nm)	AuNPs (nm)					AuNPs (nm)
<i>Mesogold</i>	3.2	6.5 $\pm$ 0.1					5.1 $\pm$ 2.4
Concen.	Teórico	HDC-ICP-MS			UF-FAAS	FAAS	FESEM
	Plata total (mg L⁻¹)	AgNPs (mg L⁻¹)	Ag(I) (mg L⁻¹)	Plata total (mg L⁻¹)	Ag(I) (mg L⁻¹)	Plata total (mg L⁻¹)	
Suplem. 1 de plata	25	4.3 $\pm$ 1.2	14.2 $\pm$ 0.5	18.5 $\pm$ 2.4	16.8 $\pm$ 0.5	21.3 $\pm$ 2.1	
Suplem. 2 de plata	10	*	10.9 $\pm$ 0.6	10.9 $\pm$ 0.6	11.6 $\pm$ 1.0	12.0 $\pm$ 1.6	
Suplem. 3 de plata	30	26.3 $\pm$ 0.3	*	26.3 $\pm$ 0.3	1.7 $\pm$ 0.6	37.5 $\pm$ 0.8	
Tamaño	AgNPs (nm)	AgNPs (nm)					AgNPs (nm)
Suplem. 1 de plata	-	23.7 $\pm$ 0.8					23.4 $\pm$ 5.8
Suplem. 2 de plata	0.6-5	11.6 $\pm$ 2.2					14.2 $\pm$ 6.7
Suplem. 3 de plata	1-100	49.7 $\pm$ 0.1					-

*No cuantificado por solapamiento de picos.

Concen.: Concentración

Suplem.: Suplemento

4. Conclusions

In this chapter, HDC-ICP-MS capability to separate different gold and silver species, focusing on the resolution and the recovery obtained. Previously, HDC coupled to ICP-MS had already been successfully utilized to characterize inorganic particles. However, until now the study of the separation of metallic nanoparticles from their corresponding ionic forms had not been thoroughly investigated.

To develop a methodology to speciate gold and silver, the mobile phase used has been optimized, adding penicillamine (0.05 mM of penicillamine for gold and 1 mM of penicillamine for silver), to ensure that the ionic species are not retained in the column and appear in the chromatograms. After the optimization of the mobile phases, the resolution values obtained between ionic species and 10 nm NPs were reasonable, between 0.7 and 0.9. Despite this, it is observed that with a particle size too small or, depending on the concentration of species, peaks could overlap, causing errors in their quantification.

Using the method developed in this chapter, it has been possible to detect the presence of NPs and ionic gold and silver species in less than 10 min, as well as the simultaneous determination of particle size and concentration of both species. The analysis of nutritional supplements confirmed the presence of both species and the possibility of quantifying them, with results for size and concentration consistent with those obtained by ICP-MS, FAAS and electron microscopy (TEM and FESEM).

Despite the limitations of the developed method, HDC-ICP-MS could be an alternative and complement other techniques used for speciation, such as SP-ICP-MS, where the detectability of NPs is greatly affected by the presence of ionic species.

4. Conclusiones

En este capítulo se ha investigado la capacidad de HDC-ICP-MS para separar distintas especies de oro y plata, poniendo especial interés en la resolución y recuperación obtenidas. Anteriormente, la técnica de HDC acoplada a ICP-MS ya se había empleado exitosamente para la caracterización de partículas inorgánicas. Sin embargo, hasta el momento el estudio de la separación de nanopartículas metálicas de sus correspondientes formas iónicas no se había investigado en profundidad.

Con el propósito de desarrollar un método que permita la especiación de oro y plata, se ha optimizado la fase móvil utilizada, añadiéndole penicilamina (en el caso del oro una concentración de 0.05 mM de penicilamina y en el caso de la plata una concentración de 1 mM de penicilamina), con el objetivo de conseguir que las especies iónicas no se queden retenidas en la columna y aparezcan en los cromatogramas. Tras la optimización de las fases móviles, se consiguieron valores de resolución entre las especies iónicas y nanopartículas de 10 nm razonables, entre 0.7 y 0.9. A pesar de ello, se observa que un tamaño de partícula demasiado pequeño o que, dependiendo de la concentración de las especies, los picos se superponen, provocando errores en su cuantificación.

Usando el método desarrollado en este capítulo, ha sido posible detectar la presencia de NPs y especies iónicas de oro y plata en algo menos de 10 min, así como la determinación simultánea del tamaño de las partículas y la concentración de ambas especies. En el análisis de suplementos nutricionales se ha confirmado la presencia de ambas especies, y la posibilidad de cuantificarlas, con resultados para el tamaño y concentración que concuerdan con lo obtenido por ICP-MS, FAAS y microscopía electrónica (TEM y FESEM).

A pesar de las limitaciones del método desarrollado, HDC-ICP-MS puede ser una alternativa y complementar a otras técnicas, como es el caso de SP-ICP-MS, donde la detectabilidad de NPs se ve afectada en gran medida por la concentración de la especie disuelta.

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Chapter 2. Study of the behaviour of a silver-based nanomaterial used for animal feeding: solubility, dissolution/degradation and *in vitro* digestion

Abstract

Silver-based nanomaterials have been proposed as additives for growth-promoting in pig feeding. Risk assessment of nanomaterials to be used as feed additives involves their physicochemical characterization, as well as their dissolution/degradation in conditions representative of the gastrointestinal tract. In this chapter, a nanomaterial consisting of metallic silver nanoparticles deposited on kaolin microparticles (Ag-kaolin) has been investigated. Results have shown that less than 40% of the silver present in the nanomaterial was solubilized in water, mainly as Ag(I). The release of silver was reduced to 6% from the feed supplemented with 0.2% (m/m) of Ag-kaolin. The dissolution/degradation of the nanomaterial through the pig gastrointestinal track was studied by an *in vitro* digestion, which consisted of three steps (oral, gastric and intestinal). Around 8% of the total silver present in the supplemented feed was bioaccessible in the intestinal step, most of it as Ag(I), with less than 0.1% as silver-containing particles. Additionally, the behaviour of standards of Ag(I) and AgNPs was studied by applying the same *in vitro* digestion protocol. The effect of the feed matrix was also considered in the different studies. ICP-MS and FAAS were used for the quantification of the total silver, whereas HDC-ICP-MS and SP-ICP-MS were applied for the characterization and quantification of the different silver species.

1. Introduction

European Food Safety Authority (EFSA) has developed a guide for the evaluation of the risks of a NM applied to feed [1], in which the characterization of the NM was considered in terms of biodistribution, speciation and quantification. These parameters are crucial to evaluate the risk that the material involves. The physicochemical properties of NPs are fundamental in this assessment. Their morphology is also important, due to the

dependence of their properties, behaviour and toxicity according to their size, form and coating [2,3].

In this work, a silver-based nanomaterial, called Ag-kaolin, has been used as a feed additive to substitute antibiotics in animal feed. Due to the prohibition by the European Union to use antibiotics as additives, the search for new substances with the same antibacterial properties was needed. Silver (both Ag(I) and AgNPs) have been used as an alternative to antibiotics due to their antibacterial properties [4,5]. The addition of AgNPs, to benefit from these properties, into the pig diet has been studied [6–8]. Fondevila et al. [7] found that low doses of AgNPs in pig feed, through the addition of silver nanoparticles adsorbed on sepiolite, reduced coliform bacteria in the intestine, resulting in an improvement in the intake and growth of weaned piglets.

The solubility and the dissolution/degradation of a nanomaterial in water are important parameters to their assessment because they provide relevant information regarding their possible exposition level [1]. EFSA defines solubilization as “the proportion of a solute in a solvent under equilibrium condition”. For the nanomaterials, the dissolution “means that the material is solubilized into individual ionic or molecular species” [9]. The solubility allows identifying NM behaviour when it contacts the animal, its biodistribution and its behaviour when it is absorbed in organs and cells [10]. Studying the dissolution rate allows us to know if, at exposition levels expected, the particles will be dissolved and, therefore, the consumer would not be exposed to the particles. The approach of studying the solubility and dissolution in water of a NM allows modelling of how the particles cross the intestinal epithelium and are absorbed by the cells [9].

The study of the behaviour of the NM inside the feed matrix was also recommended by EFSA. The presence of complex matrix and the low concentrations of NM in them, make their detection and characterization more challenging. In this case, the animal feed includes a wide range of structures, which makes impossible the separation of the NM from the matrix, just like its detection and identification.

The next step in the outline risk assessment, according to EFSA guide [1], is the investigation of *in vitro* digestion. This step consists of the study of the different

transformations suffered by the NM in similar conditions as the gastrointestinal tract, until the intestine, where the nutrients are absorbed. If the nanomaterial degrades quickly or completely after the intestinal step, it will not be considered a NM, and it would be considered as a conventional material. In that case, the regulation of assessment for conventional materials must be followed. Otherwise, the next step is the study of the genotoxicity and cell toxicity and, subsequently, the *in vivo* study. The genotoxicity and the cell toxicity of Ag-kaolin have already been studied [11], showing that the NM did not induce genetic mutations, chromosomal aberrations or DNA damage. In addition, the antibacterial activity has also been studied [12], obtaining excellent activity against a wide range of bacteria.

Referring to the exposition study of AgNPs in the digestive tract, in the bibliography it was found numerous examples of the development of protocols for the simulation of human digestion [13–20]. These models were used because they were very simple and had good reproducibility, allowing the experiment replication. Also, they reflected reality, more or less accurately [14]. In general, they consisted of three steps in which they simulated the three most important parts of the gastrointestinal tract: oral, gastric and intestinal. All of them agreed with the fact that the pH in the oral step must be approximately 7. In the gastric step, the pH was acid. Finally, in the intestinal step, the pH increased until neutral pH again. However, these pH conditions varied depending on the digestion process, if the conditions were fasted or fed [18,21,22]. In fed conditions, the stomach pH increased until 5, which caused changes in the AgNPs behaviour, changing the way that they reached the intestine [22].

Several studies about the release and transformations of AgNPs, during human *in vitro* digestion, have been reported [13,17,21–32]. In a review published recently [33], the possible transformations suffered by AgNPs during the human gastrointestinal tract were discussed. Different *in vitro* digestion protocols were collected and the fate of AgNPs was explained. Through the *in vitro* digestion process studied, the possible AgNPs transformations were aggregation and disaggregation of the nanoparticles caused by changes in the salinity or the pH. The oxidative dissolution was caused by the presence of chloride or oxygen. The sulphuration and the corona formation were other possible transformations. Besides, the necessity of studying the effect of the food matrix on the fate of AgNPs was also highlighted. Walczak et al. [13] studied the behaviour of 60 nm

AgNPs and Ag(I) during the oral, gastric and intestinal steps of a human *in vitro* digestion, adapting the protocol developed by Versantvoort et al. [34] and by Peters et al. [15]. Moreover, they studied the influence of the proteins and enzymes involved in the digestion, analysing the resulting suspensions by DLS and SP-ICP-MS. They concluded that the digestion proteins stabilised AgNPs, avoiding their dissolution in the gastric step, with the formation of aggregates. However, in the intestinal step of AgNO₃ digestion, proteins caused the apparition of particles which contained silver, sulphur and chloride, observed by SEM-EDS. In the absence of proteins, in the second step, the oxidation of NPs occurred, leading to smaller particles (20-30 nm), although, due to the size limit of detection (LOD_{size}) of SP-ICP-MS, there may be still particles with smaller diameters. Elevated temperatures, low pH and high ionic strength were known to facilitate the dissolution of AgNPs. Ault et al. [30] studied the effect of pepsin, an enzyme involved in the gastric step, on 20 and 110 nm AgNPs with two different coatings: citrate and polyvinylpyrrolidone (PVP). They observed the formation of big aggregates because of the pepsin, due to the capability of the particles to adsorb pepsin in their surface, altering the surface properties [32]. The decrease of the pH in the stomach also caused the protonation of the coating of the NPs, reduced the stabilising properties of the coating and boosted the bond between particles, through the adsorption of the enzymes in their surfaces [23,24,32]. The presence of chloride in the gastric medium, also promoted the formation of aggregates, because of the formation of AgCl. It was deposited on the particle surface, originating a layer around the particles. This prevented the repulsion between particles, presumably because of the neutral conditions on the surface and the lack of steric impediment, causing the aggregation [13,22,23,31].

Definitely, the food matrix also affects the particle behaviour during the process. The components of the food have a stabilizer effect, preventing particle aggregation in the last step [17,25,27,28]. Laloux et al. [25] observed differences in human digestion in presence/absence of food matrix for AgNPs. In presence of matrix, in the initial step, the appearance of proteins corona was noticed, which was still obtained in the intestinal step, and no aggregates were observed in the stomach. However, in absence of the matrix, no changes were observed in the oral step compared with the pristine particles. During the intestinal step, the disaggregation of NPs formed in the second step was observed.

Similarly, Ramos et al. [27] also obtained differences in AgNPs transformations in presence or absence of food, adding AgNPs to a chicken meat sample (*Nanolyse-14*) and analysing by SP-ICP-MS. In absence of food, practically 100% of the silver added was recovered. Nevertheless, most of the particles were dissolved, releasing Ag(I), and probably smaller particles too, since, because of the LOD_{size}, the distributions obtained were not complete and the smaller particles were not seen. In presence of food, only 13% of the silver was recovered and only 13% of the particles reached the intestine. Therefore, the study of silver in presence of the matrix is especially important since its behaviour changes. However, most of the studies in the bibliography consist of the addition of the different forms of silver on a food sample. No *in vitro* digestion studies of real samples of feed containing silver-based NM can be found to our knowledge. The behaviour of the silver may vary if silver standards species are added to the feed, or a complex NM is used as a feed additive.

Most of the harmonized protocols for *in vitro* digestion are aimed at human digestion simulation [14,20,35]. Nonetheless, the fate of the feed studied in this chapter is pig nutrition, so the simulation of the digestion for this animal is more suitable. Miralles et al. [36] compared the oral and gastric steps of an *in vitro* digestion protocol, developed by Brodkorb et al. [14], with an *in vivo* study with pigs. They concluded that this digestion could be appropriate to simulate the degradation of the proteins, but not all of the process was studied, they only studied until the duodenum. Montoya et al. [37] investigated the *in vitro* digestion for pigs and they compared it with the *in vivo* digestion, procuring favourable results. However, they suggested the necessity of conducting more assays, intending to obtain a more robust *in vitro* digestion method. Afterwards, Egger et al. [35] developed a model of an *in vitro* digestion for pigs with the three corresponding steps: oral, gastric and intestinal, using the protocol developed by Minekus et al. [20] as a basis, eliminating the amylase in the oral step. In a later study [38], they compared the results of the protein hydrolysis of *in vivo* and *in vitro* assays, finding that, in the end, both methods were comparable, especially in the gastric and intestinal steps. Therefore, this protocol could be used to simulate the pig *in vivo* digestion with reliability.

Following the recommendations of EFSA for the risk assessment of nanomaterials, the aim of this chapter has been the characterization of a silver-based nanomaterial (Ag-kaolin) proposed to be used as a growth promoter in pig feeding. For this purpose, the

dissolution/degradation of silver has been studied, both in water and following the *in vitro* gastrointestinal digestion protocol developed by Egger et al. [35]. The behaviour of the different silver species during the *in vitro* digestion was also studied by HDC-ICP-MS and SP-ICP-MS.

2. Materials and methods

2.1. Instrumentation

To quantify the total silver present in Ag-kaolin leachate, a Perkin Elmer FAAS, Model AAnalyst 200 (Toronto, Canada), whose description and acquisition parameters are shown in the Instrumentation section in Chapter 1 (page 86).

A Perkin Elmer NexION 2000 ICP-MS (Perkin Elmer, Toronto, Canada) in quantitative mode, with Syngistix software version 2.5, provided by the manufacturer, was also used for the analysis of the total concentration of silver of the rest of the samples. The measurement conditions used, optimized daily, are shown in table 2.1. To carry out the daily optimization of the equipment, a solution provided by the manufacturer was used, whose composition was 1 $\mu\text{g L}^{-1}$ of Be, Ce, Fe, In, Li, Mg, Pb and U in 1% HNO_3 .

Table 2.1. Conditions used in the measurement by ICP-MS.

ICP-MS NexION 2000	
Forward power (W)	1600
Argon gas flow	
Plasma (L min^{-1})	15
Auxiliary (L min^{-1})	1.2
Nebulizer (L min^{-1})	1.04
Sample flow rate (mL min^{-1})	0.35
Acquisition parameters	
Dwell time (ms)	50
Reads per replica	1
Replicas	10
Settling time (ms)	0
Monitored isotopes	^{107}Ag , ^{109}Ag
Measurement delay (s)	120
Cleaning time (s)	120

In addition, before carrying out the measurement, the silver measurement parameters were optimized daily, to obtain the maximum sensitivity for silver. This optimization was done with a solution of $1 \mu\text{g L}^{-1}$ of Ag(I) in 1% HNO_3 , prepared daily.

For the measurements by SP-ICP-MS, the same instrument Perkin Elmer NexION 2000 ICP-MS was used in single particle mode, with the Nano software version 2.4, provided by the manufacturer. The optimized measurement conditions are shown in table 2.2. Before conducting the analysis, it was necessary to obtain the transport efficiency. For this, the size procedure described by Pace et al. [39], which is detailed in the following procedures section, was used.

Table 2.2. Conditions used in the measurement by SP-ICP-MS.

ICP-MS NexION 2000	
Forward power (W)	1600
Argon gas flow	
Plasma (L min^{-1})	15
Auxiliary (L min^{-1})	1.2
Nebulizer (L min^{-1})	1.04
Sample flow rate (mL min^{-1})	0.35
SP-ICP-MS	
Dwell time (μs)	100
Readings per replicate	500 000
Settling time (ms)	0
Acquisition time (s)	50
Monitored isotope	^{107}Ag

For HDC-ICP-MS measurements, the same instrument used in Chapter 1 (Instrumentation section on page 86) was utilized. Table 1.1 in Chapter 1 (page 87) shows the experimental conditions of the ICP-MS and the HDC used. For the characterization and quantification of silver species, the composition of the mobile phase used was 0.45 mM of SDS and 1 mM of penicillamine. This mobile phase was chosen due to the compromise achieved between the recovery and the resolution between AgNPs and Ag(I), as was explained in Chapter 1 (pages 96-97) [40].

Origin 9.6.5.169 (OriginLab Corporation, MA, Northampton, U.S.A.) software was used for the representation and processing of the data.

2.2. Chemicals

AgNPs suspensions used were prepared from commercial solutions (NanoComposix, San Diego, USA). Stabilized with 2 mM citrate, they had a concentration of 21 mg L⁻¹ and nominal diameters of 10, 20, 40, and 60 nm (10.3 ± 2.1 , 18.6 ± 2.7 , 39 ± 5 , 59 ± 6 nm, respectively). Ag(I) (994 ± 3 mg L⁻¹) and Au(III) (999 ± 4 mg L⁻¹) standards (Sigma-Aldrich, St. Louis, USA) were also utilized. Rh was used as an internal standard in ICP-MS and was prepared from a standard solution with a concentration of 1002 ± 6 mg L⁻¹ (Sigma-Aldrich, Sta. Louis, USA). The solutions were prepared by weighing with an analytical balance (GH-200, A&D, California, USA).

For the preparation of the mobile phase (0.45 mM SDS and 1 mM penicillamine) for HDC measurements, DL-Penicillamine (Sigma-Aldrich, Sta. Louis, USA) and SDS (Bio-Rad Laboratories, Hercules, USA) were used. The pH of the mobile phase was 7.5, which was adjusted with solutions of HNO₃ (69/70%, J.T. Baker, Phillipsburg, USA) and NaOH (Prolabo, Barcelona, Spain), with a GLP 21 pH meter (Crison, Barcelona, Spain).

For the determination of the transport efficiency for the SP-ICP-MS measurements, a suspension of AuNPs with a nominal diameter of 47.8 ± 1.8 nm (Nanocomposix, San Diego, USA) with a concentration of 52 mg L⁻¹ (4.7×10^{10} particles mL⁻¹) as certified material in size and concentration was used. For the preparation of the solutions of the calibration curve, Au(III) standard solution (999 ± 4 mg L⁻¹) (Sigma-Aldrich, Sta. Louis, USA) was used.

The enzymes required for the *in vitro* digestion were pepsin from porcine gastric mucosa of 3200-4500 U mL⁻¹, pancreatin from porcine pancreas based on trypsin activity, and B3883 bovine bile (Sigma-Aldrich, St. Louis, USA). For the preparation of the simulated fluids, potassium chloride (KCl), potassium dihydrogen phosphate (KH₂PO₄), sodium bicarbonate (NaHCO₃) were used (Sigma-Aldrich, St. Louis, USA); sodium chloride (NaCl) (VWR Chemicals Prolabo, New Jersey, USA), magnesium chloride (MgCl₂·6H₂O) (Fisher, Leicestershire, UK), ammonium carbonate ((NH₄)₂CO₃) (Fluka BioChemika, Buchs, Switzerland) and calcium chloride (CaCl₂·2H₂O) (Fisher, Leicestershire, UK) were also utilized. NaOH (Prolabo, Barcelona, Spain) and HCl (37%, J.T. Baker, Phillipsburg, USA) were used to adjust the pH of the digestive solutions.

For the preparation of the mobile phase, the samples and the dilutions, ultrapure water (Milli-Q Advantage, Molsheim, France) was used.

2.3. Samples

The material to be studied is a nanomaterial, called Ag-kaolin, provided by Laboratorios Enosán S.L. This sample is a commercial kaolin in which AgNPs were deposited on the surface of the kaolin particles.

The concentration of silver present in the Ag-kaolin was determined by an acid attack with HNO_3 and analysed by FAAS. The concentration obtained was $8.32 \pm 0.35 \text{ mg g}^{-1}$, which corresponds to $0.83 \pm 0.04\%$ (w/w) [11]. Ag-kaolin was also analysed by FESEM, obtaining particles with a more frequent size of 30 nm, approximately (measured using the Image J software version 1.53) (see figure A2.1 and A2.2 in Appendix 2).

Ag-kaolin was added to pig feed in a proportion of 0.2%. The composition of the feed was: 34.6% of dry matter, 34.6% of organic matter, 7.69% of protein, 1.54% of exogen enzymes, 3.46% of fibre and 17.3% of starch. Two types of feed were prepared: control feed, to which kaolin without silver was added, and feed containing Ag-kaolin with a theoretical concentration of 20 mg kg^{-1} ($10 \text{ mg AgNP/g kaolin}$).

2.4. Procedures

2.4.1. Study of the solubility and dissolution rate of silver from Ag-kaolin

To determine the solubility of silver of Ag-kaolin in water, several suspensions of Ag-kaolin were made: 0.2, 0.4, and 1 g L^{-1} . These suspensions were prepared by weighing 10, 20 and 50 mg of Ag-kaolin, respectively, and suspending them in 50 mL of ultrapure water.

The samples were shaken at 99 rpm for 1 h in the dark and at room temperature. Once this time had elapsed, the suspensions were centrifuged at 7000 g for 17 min, to leave particles larger than $1 \mu\text{m}$ at the bottom of the tube. Subsequently, the supernatant and the solid were separated and the supernatant was diluted with ultrapure water to reach the optimum concentration for the analysis, which depends on the sensitivity of the instrument used. The solutions were measured by FAAS to obtain the total concentration

of solubilized silver. SP-ICP-MS and HDC-ICP-MS were used to obtain the different species originated from the dissolution/degradation of the Ag-kaolin.

EFSA Guidance [9], recommends the use of ultrafiltration to remove any possible suspended particles. In this work, ultrafilter devices with a 3 kDa separation membrane, from *Nanosep Pall*, were used to remove any particles, allowing only Ag(I) to pass through it. The solutions were analysed by FAAS. In the beginning, the devices were washed twice with 500 μ L of ultrapure water and centrifugation. Then, 500 μ L of the supernatants of Ag-kaolin suspensions were centrifugated at 15000 g for 30 min at 20 °C (Thermo Heraeus XIR centrifuge, with an Eppendorf rotor, Waltham, USA). The ultrafiltration recovery obtained was $93.3 \pm 1.6\%$.

2.4.2. Determination of the total content of silver in the pig feed

The total silver content in the feed was obtained by acid attack and subsequent analysis by ICP-MS, following the procedure described by Jiménez-Lamana et al. [41]. Three replicates were performed. 7 mL of HNO₃ and 3 mL of HCl were added to 0.1 g of feed, in Teflon microwave beakers. Subsequently, they were introduced in the microwave (Mars 6 (CEM, Charlotte, North Carolina, USA)) at 200 °C and 800 psi for 30 min. The resultant supernatants were made up with 3% of HCl (v/v) to 50 mL, to maintain the concentration of Cl⁻ in the medium and avoid the precipitation of AgCl.

Blank recovery was carried out, spiking 5 μ g L⁻¹ of Ag(I), and submitting the solution to the same protocol. Sample recovery was also performed, spiking 5 μ g L⁻¹ of Ag(I) on a control feed sample and performing the acid attack.

For the analysis by ICP-MS, a calibration curve of Ag(I) in 2% of HNO₃ and 6% of HCl (v/v) with concentrations from 0 to 15 μ g L⁻¹ was analysed. 10 μ g L⁻¹ of Rh was used as an internal standard.

2.4.3. *In vitro* digestion

The *in vitro* digestion protocol followed in this work was described firstly by Minekus et al. [20], and optimized and validated for pigs subsequently by Egger et al. [35]. Table 2.3 shows the composition and the concentrations of the corresponding salts needed to prepare the different digestive fluids: simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). The scheme of the digestion protocol used is shown in figure 2.1.

Table 2.3. Composition of the *in vitro* digestion fluids.

Salt component	Concentration (g L ⁻¹)	SSF (mL)	SGF (mL)	SIF (mL)
KCl	37.5	15.1	6.9	6.8
KH ₂ PO ₄	68	3.7	0.9	0.8
NaHCO ₃	84	6.8	12.5	42.5
MgCl ₂ ·6H ₂ O	30.5	0.5	0.4	1.1
NaCl	117	-	11.8	9.6
(NH ₄) ₂ CO ₃	48	0.06	0.5	-

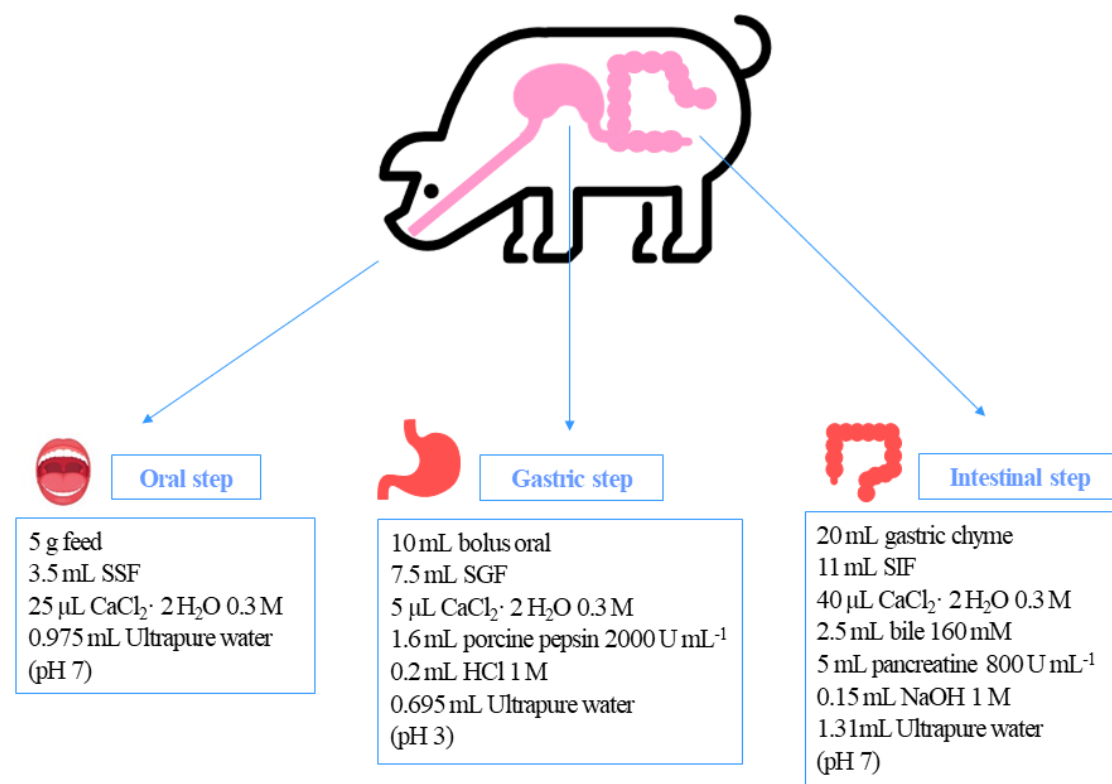


Figure 2.1. Scheme of the *in vitro* digestion process used.

For the oral step, 3.5 mL of SSF and 0.025 mL of the solution of 0.3 M of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ were added to 5 g of animal feed supplement with Ag-kaolin. Subsequently, with ultrapure water, it was filled up to 10 mL. This suspension was left in the incubator (OVAN, Barcelona, Spain) for 2 minutes at 37°C at 99 rpm. For the gastric step, the 10 mL of oral suspension was mixed with 7.5 mL of SGF, 0.005 mL of the solution of 0.3 M of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 1.6 mL of a suspension of porcine pepsin. The correct amount of 1 M HCl was added to achieve a pH of 3. Finally, ultrapure water was added to reach a volume of 20 mL. This suspension was left to incubate for 2 h at 37°C at 99 rpm. For the intestinal step, 20 mL of the gastric step was mixed with 11 mL of SIF, 0.04 mL of the solution of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.5 mL of the suspension of bile, and 5 mL of the suspension of pancreatin. Enough amount of 1 M NaOH was added to the suspension to achieve a pH of 7. Finally, ultrapure water was added to 40 mL. The suspensions were left incubating for 2 h at 37°C at 99 rpm.

After each step, and before adding the solutions for the next one, the suspensions were sonicated in an ultrasonic bath (JP Selecta, Barcelona, Spain) to homogenize them and thus, increase their reproducibility. To continue with the digestion, the corresponding solutions were added, and the procedure continued. In the case of the analysis of each corresponding step, the resulting supernatant was separated from the remaining solid by centrifugation for 17 min at 7000 g, to leave the particles with a size greater than 1 μm at the bottom. The supernatants of each step were diluted with ultrapure water, considering that only 0.1% of solids could be introduced into the instrument.

To study the behaviour of the silver species, the same *in vitro* digestion protocol was applied to Ag(I) (5 mg L⁻¹) and 20 nm AgNPs (1 mg L⁻¹) standard solutions for HDC-ICP-MS analysis and 40 nm AgNPs (0.1 mg L⁻¹) standard solutions for SP-ICP-MS analysis. To study the effect of the matrix in the digestion process of the species of silver, spikes of Ag(I) and AgNPs (0.5 mg L⁻¹) on a control feed sample were also carried out.

The different extractant solutions for each step of the digestion of feed, standards, and spiked control were analysed by ICP-MS for total silver determination and SP-ICP-MS and HDC-ICP-MS for silver species characterization.

2.4.4. Quantification of the total silver by FAAS and ICP-MS

For the quantification of the total silver present in the supernatants and in the leachate of Ag-kaolin, FAAS was used, meanwhile, ICP-MS was utilized for the total content of the leaching of the feed and the *in vitro* digestion.

For FAAS analysis, a calibration curve of Ag(I) in 1% of HNO₃, with concentrations between 0 to 5 mg L⁻¹ was measured.

For the quantification by ICP-MS, a calibration curve of Ag(I) in 1% of HNO₃, with concentrations between 0 to 15 µg L⁻¹ was analysed. A solution of 10 µg L⁻¹ of Rh in 1% of HNO₃ was used as an internal standard.

2.4.5. Characterization and quantification of silver species by SP-ICP-MS

Before the SP-ICP-MS measurements, the determination of the transport efficiency is needed. In this study, the method chosen to obtain this parameter was the particle size method [39]. Then, for the calculation of the transport efficiency, a suspension of AuNPs of 47.8 nm with the corresponding concentration (in the order of 200-250 ng L⁻¹) was used. A calibration curve for Au(III) with concentrations between 0 and 1 µg L⁻¹ in ultrapure water was employed. To obtain the value by this method, it is also necessary to know the sample flow rate of the instrument, which was obtained by the weight difference of a tube before and after 2 min of sample inlet to the instrument. The sample flow rate was 0.344 ± 0.012 mL min⁻¹.

In addition, for the determination of Ag(I) concentration, a calibration curve between 0 and 1 µg L⁻¹ of Ag(I) in 1% HNO₃ was prepared. In the analysis by SP-ICP-MS, it is necessary to discriminate between particles and baseline. To do this, 5σ was chosen as the limit of the baseline, where σ was the standard deviation of the baseline. All points that were above this limit would be considered NPs. With this criterion, it is possible to ensure a balance between the number of particle events which must be high enough so that it can be used quantitatively and ensure a decrease in false positives [42–44].

The optimal dilutions of the supernatants were calculated according to Ag(I) and silver particle concentrations so that the results were as reliable as possible. For this, an Excel

spreadsheet was used, which through the sample flow rate, transport efficiency, peak width, acquisition time and the size, density and mass fraction of the particles, the optimal dilution can be obtained [45].

2.4.6. Characterization and quantification of silver species by HDC-ICP-MS

Following the procedure described in Chapter 1, the characterization and quantification of the silver species in the supernatants of the samples were analysed by HDC-ICP-MS.

First, to obtain the calibration curve for the characterization of the size of the silver particles, suspensions of 10, 20, 40 and 60 AgNPs ($250 \mu\text{g L}^{-1}$) were measured, and the retention time was plotted against the size. For the calibration curve to quantify the silver, several solutions of Ag(I) in 1 mM penicillamine (from 50 to $350 \mu\text{g L}^{-1}$) were measured without column, and the area of the peaks was graphed against the concentrations. To calculate the concentrations of the samples, the area of the peaks was interpolated on the corresponding curve [40].

3. Results and discussion

3.1. Solubilization and dissolution/degradation of Ag-kaolin in water

Three suspensions of each ratio solid to liquid of Ag-kaolin in ultrapure water (from 0.2 to 1 g L^{-1}) were measured by FAAS, to quantify the total amount of silver released from the additive. In addition, ultrafiltration of the supernatants was carried out to obtain the concentration of Ag(I).

When Ag-kaolin was put in contact with water, a rapid sedimentation of particles over around $2 \mu\text{m}$ was observed and the remaining particles with diameters of $1 \mu\text{m}$ in the suspension were also seen within the first 30 min. Because of this, centrifugation (7000 g for 17 min) was needed firstly, to remove the particles with sizes larger than $1 \mu\text{m}$ and deposit them at the bottom of the tubes.

Due to the insolubility of the particles of kaolin, the behaviour of the silver was considered to study the solubilization of the nanomaterial in ultrapure water. The concentrations obtained for total silver and Ag(I) after solubilization, and ultrafiltration

of Ag-kaolin are in table 2.4. The total silver solubilized was around 3 mg g⁻¹, which corresponds to 35-46% of the total silver present in Ag-kaolin (8.32 mg g⁻¹).

Table 2.4. Total silver and Ag(I) determination in the supernatants from the leaching of Ag-kaolin measured by FAAS. Mean $\pm$ standard deviation ($n=3$).

Ratio of the suspension of Ag-kaolin (g L ⁻¹)	Total silver concentration (mg g ⁻¹)	Ag(I) concentration (mg g ⁻¹) (ultrafiltration)	Ag(I) vs. total silver released (%)	Total silver released vs. total silver (%)
0.2	3.28 $\pm$ 0.23	3.12 $\pm$ 0.56	86.4 $\pm$ 13.6	39.4 $\pm$ 2.8
0.4	3.80 $\pm$ 0.05	3.71 $\pm$ 0.25	97.6 $\pm$ 5.5	45.7 $\pm$ 0.6
1	2.94 $\pm$ 0.06	2.91 $\pm$ 0.16	99.0 $\pm$ 7.7	35.4 $\pm$ 0.8

When Ag-kaolin is dispersed in water, AgNPs and Ag(I), generated from the dissolution of the nanoparticles due to their oxidation or the dissolution of the coating of the particles with a composition of silver oxide [46], might be released. To determine if nanoparticles or Ag(I) was released, the supernatants from the leaching of Ag-kaolin were submitted to ultrafiltration (with a membrane with 3 kDa of pore size) and then analysed by FAAS. The results of the ultrafiltration (table 2.4) show that most of the silver was released as Ag(I), more than 86% of the total silver depending on the solid-to-liquid ratio, being up to 99% for the 1 g L⁻¹ suspension.

3.1.1. Characterization of silver species solubilized from Ag-kaolin by SP-ICP-MS

Supernatants from Ag-kaolin suspensions of 0.2, 0.4 and 1 g L⁻¹ were measured after adequate dilution in ultrapure water. Only results from the suspension of 1 g L⁻¹ of Ag-kaolin are shown in table 2.5 since no particles could be detected in the suspensions of 0.2 and 0.4 g L⁻¹. The total silver released for the suspension of 1 g L⁻¹ of Ag-kaolin measured by SP-ICP-MS was 2.99 mg g⁻¹, in agreement with the concentration obtained by FAAS. The percentage of dissolved silver was also in agreement with the result obtained by ultrafiltration (99.0% of the silver was released as Ag(I)). The precision between samples ($n=4$) was quite good in terms of the concentration of Ag(I) obtained, with DSR values around 5-6%. However, for the concentration of silver particles, the precision was lower (DSR value of 11%). This is because of the low concentration of silver particles obtained in the supernatant, around 1 ng L⁻¹, which corresponds to less

than 0.1% of the total silver released. This means that most of the silver that was released in water was dissolved silver.

Table 2.5. Characterization and determination of the silver released from the supernatant of Ag-kaolin leaching by SP-ICP-MS. Mean $\pm$ standard deviation ($n=4$).

Ratio of the suspension of Ag-kaolin (g L^{-1})	Most frequent diameter (nm)	Ag(I) concentration released (mg g^{-1})	AgNPs concentration released (ng g^{-1})	Total silver concentration released (mg g^{-1})	Total silver released vs. total silver (%)
1	30 ± 3	2.99 ± 0.23	1.08 ± 0.12	2.99 ± 0.23	35.9 ± 2.7

Figure 2.2 shows the mass per particle distribution obtained for 1 g L^{-1} Ag-kaolin suspension. Due to the low number of events recorded with a time of acquisition of 50 s (100 events), the sample was analysed for 5 min, obtaining the distribution shown in figure 2.2. Even so, the number of events obtained was approximately 570, confirming the low silver-containing particle concentration released from Ag-kaolin.

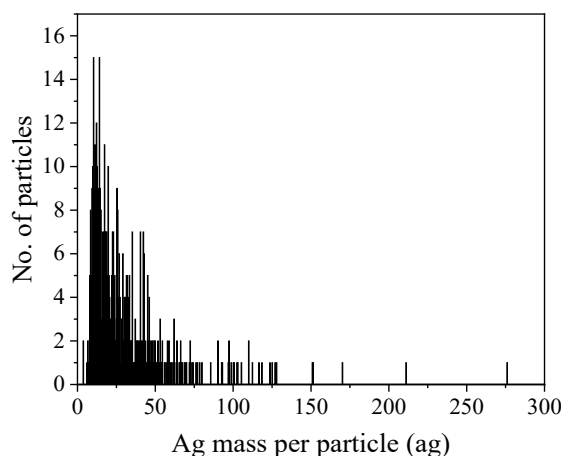


Figure 2.2. Silver mass per particle distribution by SP-ICP-MS for 1 g L^{-1} Ag-kaolin leaching.

Due to the presence of a high content of dissolved silver, the mass per particle limit of detection (LOD_{mass}) has to be considered [47,48]. With high dissolved species concentration, the LOD_{mass} would be high, since the smaller particles will be masked by the baseline noise. Thus, larger real-size values and a lower particle concentration will be obtained [49]. In the supernatants of Ag-kaolin, the LOD_{mass} , calculated following equation A.1 (Appendix 1), was 6 ag. This value would correspond to a size of 19.6 nm.

3.1.2. Characterization of silver species solubilized from Ag-kaolin by HDC-ICP-MS

The supernatant of the suspension of 1 g L^{-1} of Ag-kaolin in water was also analysed by HDC-ICP-MS using a mobile phase with a composition of 0.45 mM SDS and 1 mM penicillamine. The chromatogram obtained is shown in figure 2.3. A single peak appeared with a retention time of $8.74 \pm 0.01 \text{ min}$, which corresponds to Ag(I) (the retention time of the standard in water was $8.82 \pm 0.02 \text{ min}$). These results agree with the data obtained by SP-ICP-MS (table 2.5), in which the concentration of silver particles was very low ($1.08 \pm 0.12 \text{ ng g}^{-1}$), below the LOD of HDC-ICP-MS ($0.041 \text{ } \mu\text{g L}^{-1}$). The Ag(I) concentration obtained ($2.80 \pm 0.11 \text{ mg g}^{-1}$) was comparable with the one obtained by SP-ICP-MS ($2.93 \pm 0.23 \text{ mg g}^{-1}$) and FAAS ($2.91 \pm 0.16 \text{ mg g}^{-1}$). The concentration obtained corresponds to $33.7 \pm 1.3\%$ of the total silver present in the nanomaterial is solubilized.

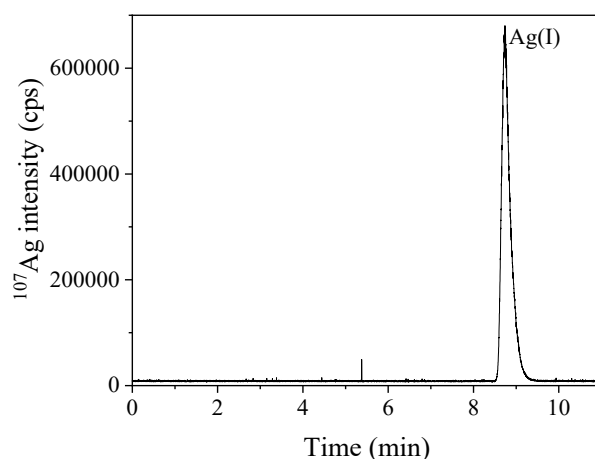


Figure 2.3. HDC-ICP-MS chromatogram obtained from the supernatant of a suspension of 1 g L^{-1} of Ag-kaolin in water.

The study for the solubilization and dissolution/degradation of the feed supplemented with Ag-kaolin was also studied and the results are presented in Appendix 2 (page 264). Only 6% of the total silver present in the supplemented feed was solubilized in ultrapure water after 24 h. The silver released from the feed was lower than from the Ag-kaolin itself (around 35-40%). The supernatants of the feed leachate were analysed by SP-ICP-MS, obtaining that most of the silver released was also Ag(I) (99.9%).

3.2. Total silver in supplemented pig feed

The results obtained for the total silver content measured by ICP-MS and the recoveries for the procedure and sample are shown in table 2.6. The silver content in the supplemented feed was $12.6 \pm 1.1 \text{ mg kg}^{-1}$, whereas no silver was detected in the non-supplemented one. Recoveries of the procedure were better than 88%.

Table 2.6. Total silver concentration in pig control and supplemented feed. Mean $\pm$ standard deviation ($n=6$).

Sample	Silver results
Control feed	<LOQ
Feed supplemented with Ag-kaolin	$12.6 \pm 1.1 \text{ mg kg}^{-1}$
Procedure recovery	$98.0 \pm 3.4 \%$
Control feed recovery	$88.2 \pm 13.9 \%$

LOQ: $0.012 \text{ } \mu\text{g kg}^{-1}$

3.3. *In vitro* digestion of the supplemented pig feed

Following the second step proposed by the EFSA Guidance [1], after the study of the solubilization of the nanomaterial, the *in vitro* digestion study was performed.

The feed supplemented with Ag-kaolin was subjected to the three steps of the *in vitro* digestion (oral, gastric and intestinal) following the procedure described in the previous section (section 2.4.3). Each step was studied separately to determine the amount of silver released and the different silver species present in the supernatants: as free metallic nanoparticles, as nanoparticles adsorbed on kaolin microparticles with sizes below $1 \text{ } \mu\text{m}$, as Ag(I), generated from the oxidation of the nanoparticles, or as other species generated from the different transformations suffered through the *in vitro* digestion, such as AgCl-NPs, aggregates of silver nanoparticles, or other silver-containing particles.

According to the EFSA guide [1], only the results of the intestinal step are considered relevant. However, it also argues that the first two steps are necessary to simulate the intestinal step and they are relevant in terms of the study of the transformations of the NM during the digestion process, although these data are not required for the evaluation.

3.3.1. Determination of the total silver released through the *in vitro* digestion by ICP-MS

The supernatant from each step of the *in vitro* digestion was collected and analysed by ICP-MS. Before the analysis of the samples, the slopes of the calibration curves prepared with the fluids of each digestion step and the one prepared in 1% of HNO₃ were compared with a Student's *t*-test. The results of the slopes, their corresponding deviation and the *t* calculated for each of them, are listed in table 2.7. The reference *t* is 2.92, with a confidence value of 90%. There were no significant differences between the slope of the calibration in 1% HNO₃ and those in the digestive fluids, therefore the calibration in 1% HNO₃ was used for the quantitative determination of silver in the different samples.

Table 2.7. Slopes, deviations and calculated *t* for the calibration curves by ICP-MS. *t*_{ref}: 2.92.

Medium	Slope	Deviation	<i>t</i> _{cal}
1% HNO ₃	0.210	0.0027	-
SSF	0.203	0.0008	2.39
SGF	0.217	0.0038	1.46
SIF	0.260	0.0207	2.37

The concentration of total silver released in each step, as well as the percentage, regarding the total silver present in the feed, is shown in table 2.8. In the oral and gastric steps, less than 2% of the total silver was released. In the intestinal step, the percentage of total silver that was released exceeded 8%. This percentage is the amount of silver that was bioaccessible. Bioaccessibility is described as the amount of compound that is released from the food matrix into the gastrointestinal tract and available for absorption in the intestine [50]. In other words, around 8% of the total silver present in the feed was released in the intestine and was available for subsequent absorption. A slight decrease in the silver obtained in the supernatant was observed in the gastric step. The low pH and the presence of chloride ions are the cause of this decrease, by promoting the formation and precipitation of AgCl [8,23,51]. The increased mobilization of silver in the intestinal step could be explained by the oxidation processes of the AgNPs on the surface of the kaolin particles [24] or the solubilization of the AgCl formed during the second step.

Table 2.8. Determination of the total silver released during the *in vitro* digestion by ICP-MS. Mean $\pm$ standard deviation ($n=3$).

Step	Total silver released ($\mu\text{g g}^{-1}$)	Total silver released vs. total silver (%)
Oral	0.232 ± 0.008	1.84 ± 0.07
Gastric	0.224 ± 0.067	1.78 ± 0.53
Intestinal	1.05 ± 0.01	8.33 ± 0.01

To follow the transformations suffered by silver through *in vitro* digestion, the supernatants were analysed by SP-ICP-MS and HDC-ICP-MS. By SP-ICP-MS, information about particulate forms (which might include Ag-kaolin particles) was collected, meanwhile by HDC-ICP-MS, discrimination between dissolved and silver particles was obtained.

3.3.2. Characterization of silver species through the *in vitro* digestion by SP-ICP-MS

The supernatants of each step were analysed by SP-ICP-MS, to ensure the presence or absence of particles. The low LOD of this technique makes it suitable for the analysis of the samples, due to the low particle concentration expected.

For the analysis, a calibration curve of 1% of HNO_3 was used since there were no significant differences after the realization of a *Student's t test* of the calibration curves of the digestive fluids and HNO_3 .

For every step, the concentrations of Ag(I) and silver particles, as well as their release rate and the equivalent size are presented in table 2.9. Most of the total silver released in all steps was as Ag(I). The number of events for the silver particles was very low (500 events for the oral and gastric steps and 100 events for the last step, which actually corresponds to a particle concentration below the LOQ (1.43×10^6 particles L^{-1}), (calculated following the equation A.3 in Appendix 1)), even increasing the acquisition time to 5 minutes. This represents around $1 \times 10^{-5} \%$ of the total silver that was mobilized.

Table 2.9. Determination of silver species released during the *in vitro* digestion by SP-ICP-MS. Mean $\pm$ standard deviation ($n=3$).

Step	Ag(I)		Silver-containing particles		
	Ag(I) ($\mu\text{g L}^{-1}$)	Ag(I) released vs. total silver released (%)	Equivalent most frequent size (nm)	Silver particles (ng L^{-1})	Silver particles released vs. total silver released (%)
Oral	0.264 ± 0.024	99.9 ± 0.01	32 ± 1	0.0013 ± 0.0001	$5.04 \times 10^{-4} \pm 4.53 \times 10^{-5}$
Gastric	0.300 ± 0.061	99.9 ± 0.01	29 ± 1	0.0022 ± 0.0001	$7.32 \times 10^{-4} \pm 7.86 \times 10^{-6}$
Intestinal	1.69 ± 0.14	99.9 ± 0.01	-	<LOQ	-

LOQ: 1.43×10^6 particles L^{-1}

In figure 2.4, the silver mass per particle distributions for each step of the *in vitro* digestion are shown. The relative frequency is represented due to the different dilutions made for the different steps. In general, in all steps, it was observed that the most frequent silver mass per particle obtained was less than 150 ag. This would correspond to an equivalent hydrodynamic diameter of 30 nm, approximately, which was the most frequent equivalent size obtained in TEM images (figure A2.2 in Appendix 2). However, it cannot be ensured that the particles that appear in the steps were the initial particles added since the particles can change their properties during the digestion process [25,33]. It was observed that the concentration of particles increased slightly in the second step but decreased again in the third one. This decrease can be explained by the binding of the particles to the enzymes that are present in the intestine (bile and pancreatin) and the increase in the pH of the medium to 7. With the addition of these enzymes, heteroaggregates with proteins, with a size larger than 1 μm , could be formed [21,24,26–28], which would be lost during the centrifugation.

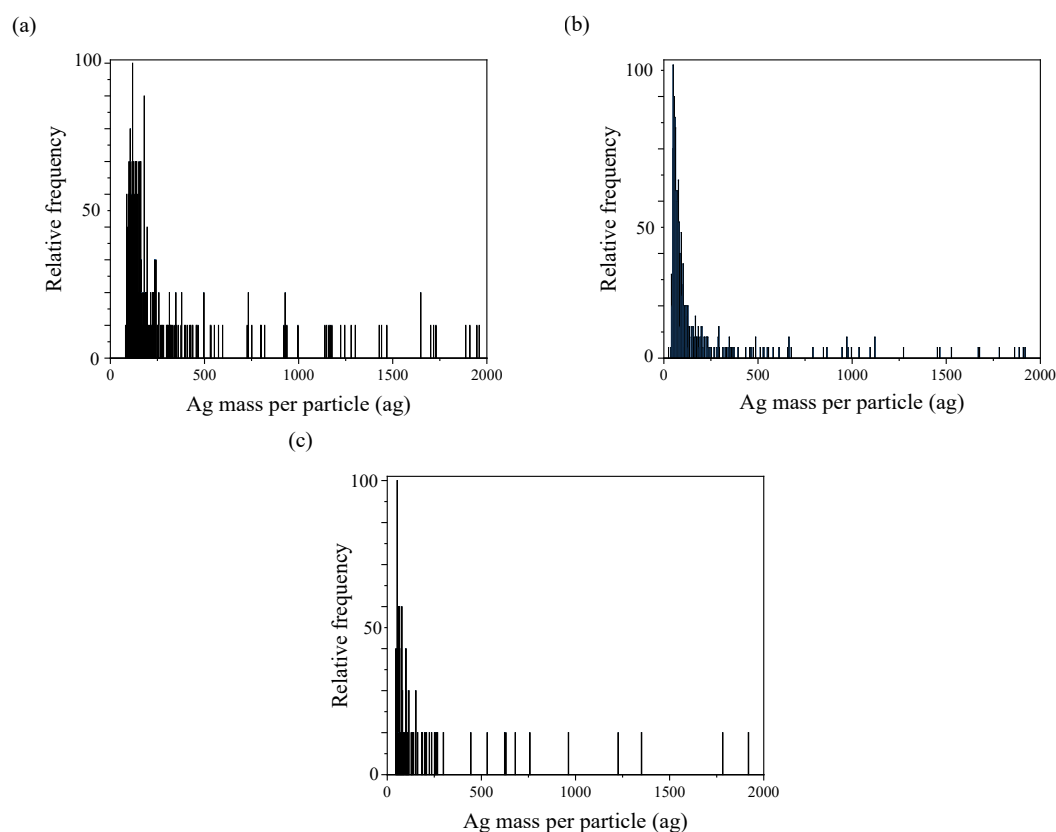


Figure 2.4. Silver mass per particle distributions for every step of the *in vitro* digestion obtained by SP-ICP-MS. (a) Oral step. (b) Gastric step. (c) Intestinal step.

3.3.3. Characterization of silver species through the *in vitro* digestion by HDC-ICP-MS

The supernatants of each digestion step were analysed by HDC-ICP-MS according to the procedure explained in section 2.4.6.

Previously, the effect of the simulated digestion fluids on the slope of the calibration curves of the mass concentration of Ag(I) was studied. The slopes of the three calibration curves obtained with the digestion fluids were compared with the slope obtained with 1 mM of penicillamine (necessary to determine Ag(I) quantitatively by HDC-ICP-MS, see Chapter 1). The slopes, their deviations and the *t* calculated were shown in table 2.10. The *t* reference was 2.92, with a limit of confidence of 90%. Just for the salivary fluid, the *t* calculated was higher than the *t* reference, and, therefore, was significantly different from the calibration curve in 1 mM of penicillamine. Then, for the determination of the

concentration of silver for the two last steps, the calibration curves with 1 mM of penicillamine can be used.

Table 2.10. Slopes, deviations of the slopes and t calculated for the calibration curves of intensity versus Ag(I) concentration by HDC-ICP-MS. t_{ref} : 2.92.

Medium	Slope	Deviation	t_{cal}
1 mM PA	5.01×10^7	2.63×10^6	-
SSF	6.20×10^7	3.16×10^5	4.58
SGF	4.92×10^7	1.31×10^6	0.33
SIF	5.29×10^7	5.78×10^5	1.04

The chromatograms obtained for each step are shown in figure 2.5. In the first two steps (in black the oral step and in red the gastric step), a broad peak appeared (with maxima at 9.00 min and 9.25 min), which corresponds to Ag(I). No peak corresponding to AgNPs was obtained. This agrees with the results of SP-ICP-MS (table 2.9), where the concentration of the particles was very low, below the LOQ found by HDC-ICP-MS. In the case of the intestinal step (in blue), apart from a broad peak at 8.97 min, similar to the previous ones, another small peak at 8.40 min was present. Figure 2.6 shows the chromatograms corresponding to Ag(I) in 1 mM of penicillamine (in red), Ag(I) from the intestinal step after in vitro digestion (in blue) and the supernatant from the intestinal step of a control feed spiked with Ag(I) (in black). As it can be seen, whereas the digestion fluids did not affect the retention time of Ag(I), the feed matrix did, delaying the elution of the species and dealing with a more complex elution of Ag(I) species, with a profile resembling the chromatogram obtained in figure 2.5. In summary, by HDC-ICP-MS no silver particles were found in any of the digestion steps since, only Ag(I) species, in accordance with the results obtained by SP-ICP-MS (table 2.9), since the particulate silver concentration was below the LOQ ($15.5 \mu\text{g L}^{-1}$).

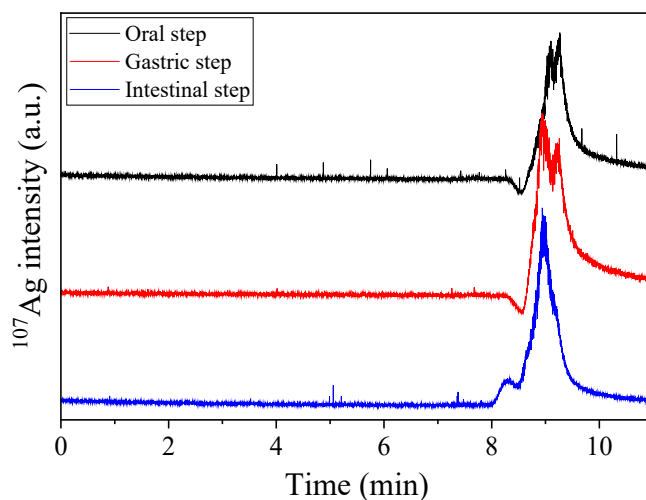


Figure 2.5. HDC-ICP-MS chromatograms from supernatants of each step of the supplemented feed *in vitro* digestion.

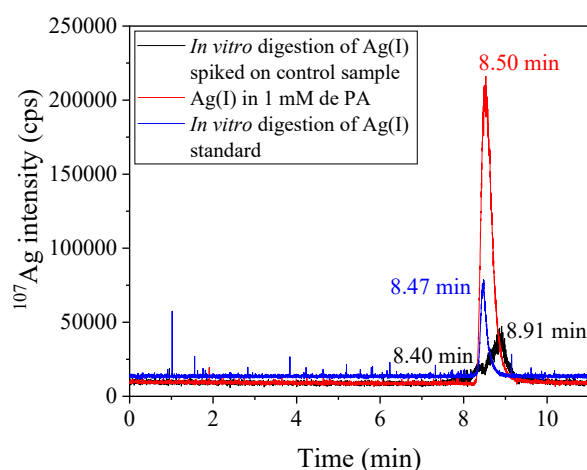


Figure 2.6. HDC-ICP-MS chromatograms for the Ag(I) standard in 1 mM penicillamine (red), in intestinal fluid (blue) and the supernatant of the intestinal step from the Ag(I) spiked control feed (black),

The concentrations of total silver released in each of the steps and its percentage with respect to the total silver in the feed are shown in table 2.11. The results are similar to the ones obtained by the rest of the techniques utilized, around 9% of the total silver in feed was bioaccessible in the intestine.

Table 2.11. Concentration of total silver released and its percentage to the total silver in feed for each step of the *in vitro* digestion measured by HDC-ICP-MS. Mean $\pm$ standard deviation ($n=3$).

Step	Total silver released ($\mu\text{g g}^{-1}$)	Total silver released vs. total silver (%)
Oral	0.273 ± 0.021	2.20 ± 0.17
Gastric	0.405 ± 0.053	3.26 ± 0.34
Intestinal	1.11 ± 0.46	8.95 ± 0.48

3.3.4. Behaviour and fate of Ag(I) and AgNPs during the *in vitro* digestion

To understand more clearly the results obtained in the *in vitro* digestion of the supplemented feed, Ag(I) (5 mg L^{-1}) and 20 and 40 nm AgNPs (0.1 mg L^{-1}) standards, as well as the corresponding spikes on the control feed, were subjected to the same digestion protocol. The supernatants, which correspond to the liquid phase and, therefore, would correspond to the bioaccessible fraction, were measured by ICP-MS to quantify the total concentration of silver released, and by HDC-ICP-MS to study the behaviour and transformations suffered by the different silver species. Since with HDC-ICP-MS, the lowest concentrations of the particles would not be visible, the supernatants were also analysed by SP-ICP-MS, to ensure the presence of particles.

The recovery percentages obtained for the silver species in absence of the feed matrix are shown in table 2.12. For both species, it was observed that more than 70% of the silver added was recovered in the intestinal step. In the case of the *in vitro* digestion of Ag(I), the recovery in the first step was quantitative. Meanwhile, in the second step, the recovery decreased to around 30%. This is due to the presence of chloride ions, which lead to the precipitation of Ag(I) as AgCl which would remain in the solid phase [21,31]. Nevertheless, in the intestinal step, the recovery increases to 81%, because of the contribution of the present enzymes to the dissolution of the precipitated silver. Similar results were found in the bibliography [8], where practically 100% of Ag(I) was recovered in the intestinal step. For the *in vitro* digestion of AgNPs standard, during the first step, practically all AgNPs were recovered. In the gastric step, the recovery decreased, because of the acid pH and the presence of chloride, which led to the oxidative dissolution of the nanoparticles and the precipitation of Ag(I) as AgCl, as well as the heteroaggregation of

the AgNPs with AgCl [23,27]. In the third step, most of the AgNPs were recovered again [13,24,25].

Table 2.12. *In vitro* digestion recoveries (%) from the liquid phase of Ag(I) and AgNPs standards by ICP-MS. Mean $\pm$ standard deviation ($n=3$).

Standard	Step	Total silver recovery (%)
Ag(I)	Oral	105 $\pm$ 8
	Gastric	31.2 $\pm$ 1.8
	Intestinal	80.8 $\pm$ 1.6
AgNPs	Oral	87.1 $\pm$ 5.7
	Gastric	68.0 $\pm$ 18.1
	Intestinal	71.5 $\pm$ 3.0

For the *in vitro* digestion of Ag(I) standard, only one peak was observed by HDC-ICP-MS (figure 2.7), which corresponds to Ag(I). In the gastric step (in red), the retention time was slightly different from the other steps. The presence of the different salts and enzymes causes a different behaviour of the silver during the separation process, which could be a cause of this delay.

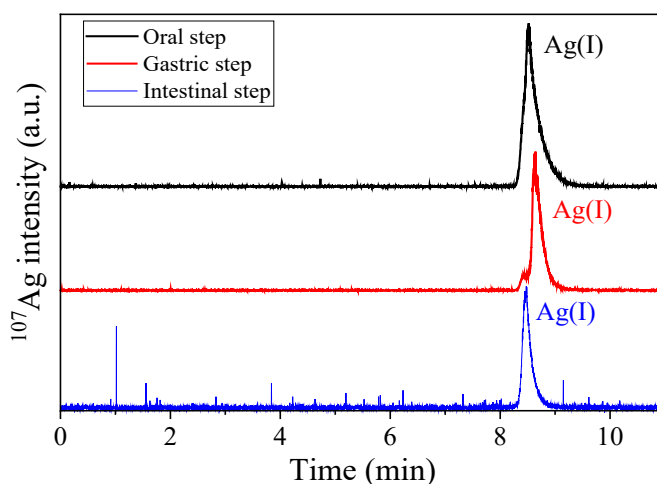


Figure 2.7. HDC-ICP-MS chromatograms from the liquid phase of the *in vitro* digestion of Ag(I) standard.

In addition, by SP-ICP-MS, some residual particles of the solid were detected, although their concentration was below the LOQ (1.42×10^6 particles L^{-1}). The presence of these particles is a consequence of the precipitation of the silver due to the presence of chloride and the acid pH, which was reported in the bibliography [8,52].

For the behaviour study of AgNPs during the *in vitro* digestion, 40 nm AgNPs were used for SP-ICP-MS and 20 nm AgNPs for HDC-ICP-MS, considering the better recovery for smaller sizes in HDC-ICP-MS (see Chapter 1 page 96). In the case of SP-ICP-MS, the LOD_{size} (15 nm), would not allow to obtain the complete distribution of 20 nm nanoparticles.

By SP-ICP-MS, a suspension of AgNPs ($0.1 \text{ mg } L^{-1}$) was submitted to the *in vitro* digestion. The distributions obtained are in figure 2.8. The most frequent size obtained for all the steps remained unchanged, 40 nm approximately. The recovery of the particles from the liquid phase in the intestinal step by SP-ICP-MS was $69.7 \pm 2.2 \%$. Nevertheless, an increase in the concentration of the baseline was detected, which means that there was a dissolution of the particles, which is also observed by HDC-ICP-MS (figure 2.9). In the chromatogram of the second step (figure 2.9 in red), the disappearance of AgNPs peak and the appearance of a single peak at 8.58 min, which corresponds to Ag(I), was observed. In the intestinal step (figure 2.9 in blue), two peaks appeared, one at 8.51 min, which corresponds to Ag(I), and another, at 8.03 min, which corresponds to AgNPs with a size of 20 nm. This means that AgNPs were recovered again. This might be because, in the second step, AgNPs heteroaggregate due to the presence of chloride [26,51], forming particles larger than $1 \text{ }\mu\text{m}$ [21,24,26–28], which would be lost in the centrifugation. In the third step, with the presence of silver complexing enzymes, redissolution of silver chloride may lead to the release of aggregated particles and would appear again in the chromatogram, with the consequent appearance of the peak at 8.03 min.

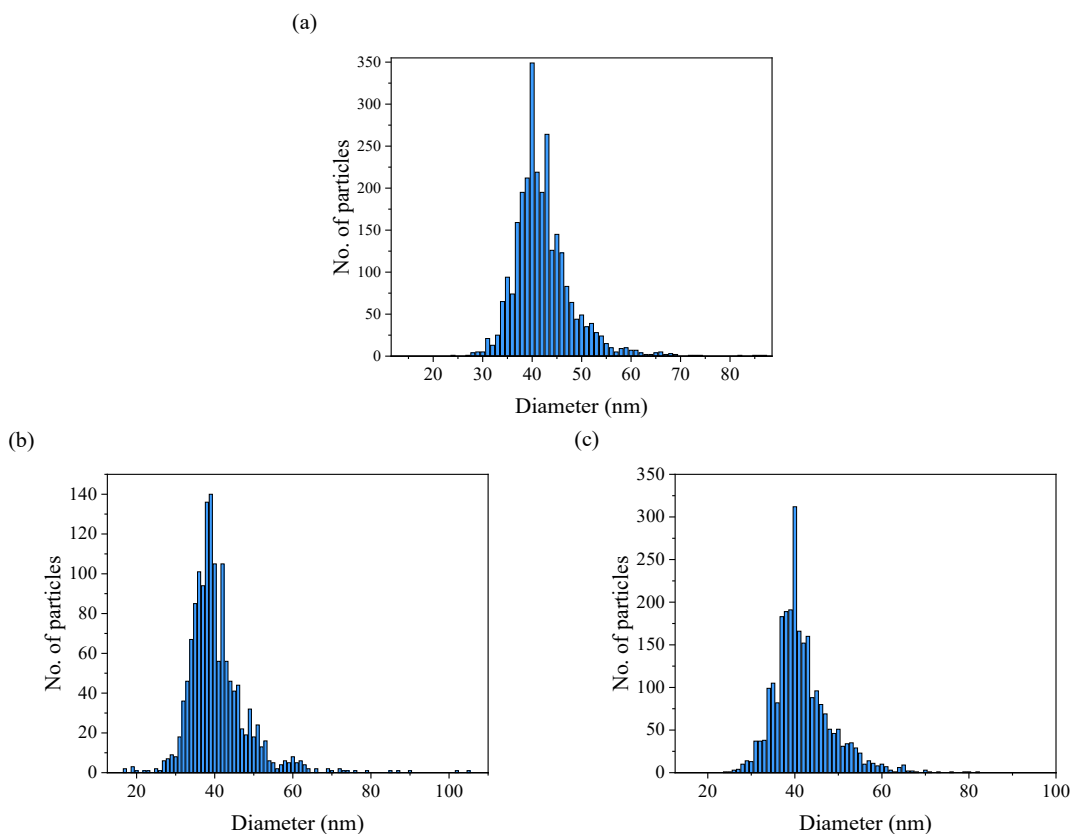


Figure 2.8. Size distributions from the liquid phase of the *in vitro* digestion of suspensions of AgNPs standard by SP-ICP-MS. (a) Oral step (b) Gastric step (c) Intestinal step.

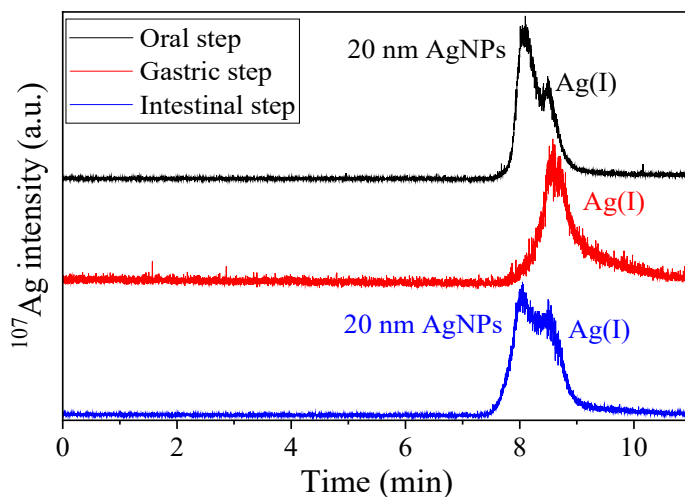


Figure 2.9. HDC-ICP-MS chromatograms from the liquid phase of the *in vitro* digestion of 20 nm AgNPs standard (1 mg L^{-1}).

According to the EFSA guide [1], the study of the impact of the components of the food in the transformations of the nanomaterial is recommended, because its behaviour could

vary in presence and absence of the food matrix. The components of the feed could cause retentions of silver in the solid matrix, which would avoid its release to the digestive medium and, therefore, would prevent its absorption in the intestine. In the bibliography, the importance of the study of the behaviour of the standards in the presence of the matrix is remarked, due to the effect caused by the food on the behaviour of the silver during the digestion process [19,27]. For this reason, Ag(I) and 20 nm AgNPs were added to the control unsupplemented feed and the corresponding digestion steps were carried out and supernatants obtained were analysed by ICP-MS (table 2.13) and HDC-ICP-MS (figure 2.10 for Ag(I) and figure 2.11 for AgNPs). The behaviour of the silver species was different in the presence of the feed matrix. The recovery of the standards spiked on the control sample was much lower with differences between both species. In the case of Ag(I), around 8% was recovered at the end of the *in vitro* digestion, while the recovery increased up to 80% for AgNPs. The Ag(I) bounds more strongly to the feed matrix [26], remaining in the solid, whereas this is not the case for AgNPs.

Table 2.13. *In vitro* digestion recoveries of Ag(I) and AgNPs spiked on control feed by ICP-MS. Mean $\pm$ standard deviation ($n=3$).

Sample	Step	Total silver recovery (%)
Ag(I) spiked on control	Oral	13.2 ± 1.2
	Gastric	7.28 ± 0.23
	Intestinal	7.99 ± 0.10
AgNPs spiked on control	Oral	12.3 ± 1.1
	Gastric	7.9 ± 1.0
	Intestinal	83.2 ± 5.1

Observing the HDC-ICP-MS chromatograms, in the case of Ag(I) (figure 2.10), broad peaks were observed in the first (in black) and second (in red) steps. The retention times of these peaks (around 9 min) corresponded to the retention times of Ag(I). In the intestinal step (in blue), other two peaks were obtained, one at 8.89 min and a shoulder at 8.40 min, which would correspond to two different species of Ag(I). This last chromatogram was similar to the one obtained for the *in vitro* digestion of the feed with Ag-kaolin (see figure 2.5), suggesting the occurrence of Ag(I) bound to small feed particles present in the supernatant.

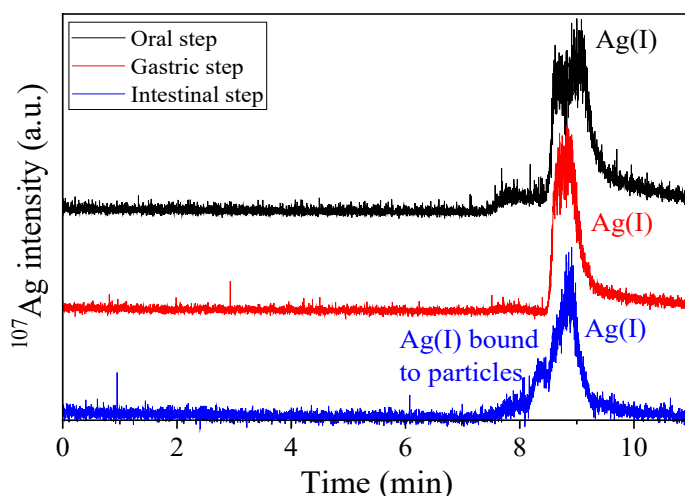


Figure 2.10. HDC-ICP-MS chromatograms obtained from the liquid phase of the *in vitro* digestion of Ag(I) spiked on feed control (5 mg L^{-1}).

For 20 nm AgNPs spiked on the control feed (figure 2.11), the chromatograms obtained for its *in vitro* digestion were like those obtained for Ag(I) spiked on the control (figure 2.10). A broad peak corresponding to Ag(I) appeared in the oral (figure 2.11 in black) and gastric steps (figure 2.11 in red), and two peaks in the intestinal step (figure 2.11 in blue). The retention times were similar (8.32 min and 8.95 min) to the times found for Ag(I) spiked (figure 2.10) and the feed sample (figure 2.5). Thus, the oxidation of nanoparticles and the consequent formation of Ag(I) were already observed in the first step, with low recovery rates in the oral and gastric steps. This might be due to the bound of part of Ag(I) to the feed matrix, which would remain in the solid fraction after the centrifugation.

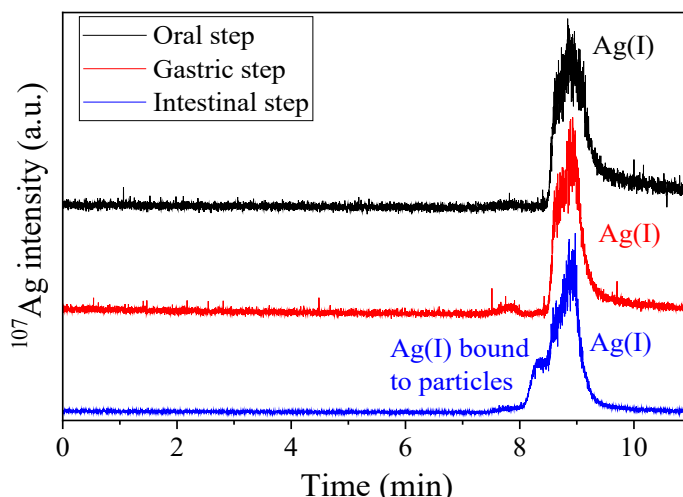


Figure 2.11. Chromatograms obtained by HDC-ICP-MS from the liquid phase of the *in vitro* digestion of 20 nm AgNPs spiked on feed control (1 mg L^{-1}).

In the bibliography, the disappearance of pristine nanoparticles during *in vitro* human digestion, especially in the stomach, was also reported [21,26,27,54]. Bove et al. [26] observed that the dissolution was practically complete (more than 90%). Even so, these ions were not available, because they bound to the digestive and feed matrix (98%), meanwhile, the rest were free dissolved ions and soluble silver complexes which would be bioaccessible.

So, the difference between the results obtained for the *in vitro* digestion of the standards with and without matrix is evident, confirming the effect of the feed matrix on the fate of the silver and the necessity of the study of its effect [33]. In the case of Ag(I), differences in the recovery of the intestinal step were also observed. For the digestion of standards without matrix, practically all the concentration added was recovered, but on the other hand, for Ag(I) spiked on control, only 8% of Ag(I) added was recovered, due to the bound of silver to feed particles. In the case of AgNPs, while in the direct digestion, particles appeared in the oral step; in AgNPs spiked on the control feed, the particles were already dissolved in the first step. For this reason, in the intestinal step, only Ag(I) appeared in the case of AgNPs spiked, meanwhile, for *in vitro* digestion of standards without matrix, the particles were recovered again. However, concerning the recovery values, they were similar, for AgNPs standard, the value was 71%, whereas for the control spiked with AgNPs, the recovery was approximately 80%.

Another significant difference between the standards and the spiked control sample was the shift of the retention times, which was also reported in the *in vitro* digestion of the feed. Thereby, it is deduced that the feed matrix displaces the retention times, not the digestive fluids because, for *in vitro* digestion of standards without matrix, the retention times were the same as the times of the standards without *in vitro* digestion.

4. Conclusions

Following the EFSA guide, the evaluation of a silver-based NM (Ag-kaolin) used as a growth promoter additive in pig feed has been carried out. The behaviour of the nanomaterial in terms of solubility and degradation/dissolution in water was investigated. An *in vitro* digestion protocol was applied to the feed supplemented with the nanomaterial. The fate and transformations of the different silver species were studied by ICP-MS, SP-ICP-MS and HDC-ICP-MS.

The study of the solubility of Ag-kaolin has shown that 30% of the total silver was released in water, most of it as Ag(I). Less than 0.01% was released as silver-containing particles. Due to the limits of detection achieved (ca. 20 nm, corresponding to 10 ag), the occurrence of particles containing lower amounts of silver could not be discarded, and therefore the percentage of particles released could be higher. In the case of the feed supplemented with Ag-kaolin, only 6% of the total silver was released in water, mostly as Ag(I).

The *in vitro* digestion of the supplemented feed has shown that around 8% of the total silver was bioaccessible, mostly as dissolved forms of Ag(I) (99.9%). Silver-containing particles could not be detected by HDC-ICP-MS, whereas less than 0.01% of silver was detected in particulate form by SP-ICP-MS. As EFSA requires that 12% (mass-based) of a NM to be present as nanoparticles after 30 min of intestinal digestion for the nano-specific risk assessment, the Ag-kaolin additive would be outside the scope of such recommendation.

The study of the behaviour of the silver standards (Ag(I) and AgNPs) during the *in vitro* digestion, showed that Ag(I) was highly bioaccessible, and the formation of particulate

silver was not observed. However, bioaccessible Ag(I) decreased to 8% in the presence of the feed matrix. For AgNPs, partial oxidation of the nanoparticles was observed, which was complete in the presence of the feed matrix. Both cases reveal the importance of carrying out such studies under feeding conditions.

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Capítulo 3. Acumulación y eliminación de plata en cerdos alimentados con pienso suplementado con un nanomaterial a base de plata. Estudio comparativo de métodos de extracción de especies de plata en heces

Part of the results of this chapter is published in the article *Comparative study of extraction methods of silver species from faeces of animals fed with silver-based nanomaterials*, Microchimica Acta, 190:204, 2023 (<https://doi.org/10.1007/s00604-023-05777-0>) ([Appendix 4](#)).

Abstract

Risk assessment of nanomaterials to be used as feed additives also involves *in vivo* experiments to study the absorption, distribution, accumulation and elimination of the nanomaterial in the animals. In this chapter, the accumulation of silver in different tissues (muscle, liver and kidney) and its elimination in faeces and slurries have been studied by analysing samples from an *in vivo* experiment with pigs fed with feed supplemented with the Ag-kaolin additive. For this purpose, total silver was analysed in tissues, faeces and slurries by ICP-MS after acid digestion. The results obtained showed that silver did not accumulate in the muscle, but it did in the liver and kidney, although most silver consumed was eliminated through faeces. Due to the use of slurries as fertilizers, and the potential mobilization of silver in the environment, different extraction methods were evaluated for the fractionation of silver species in faeces since slurries were not ultimately available. Water, tetramethylammonium hydroxide (TMAH) and a sequential extraction based on tetrasodium pyrophosphate (TSPP), in combination with ammonia and sodium sulphide, were studied. SP-ICP-MS, HDC-ICP-MS and AF4-ICP-MS were applied for the detection of particulate and dissolved silver in the extracts. The results showed that water extraction was a suitable option to assess the environmental release of silver, which accounted for less than 3% of the total silver present in faeces. TMAH allowed the simultaneous extraction of Ag(I) and silver-containing particles, nonetheless, a low extraction efficiency was obtained (42%), being a suitable choice for the detection of silver-containing particles. The TSPP-based sequential extraction allowed the

fractionation of silver into soluble silver and particulate metallic silver, silver chloride and silver sulphide, obtaining that particulate silver sulphide (45%) and soluble silver (34%) were the most abundant fractions.

Resumen

La evaluación del riesgo asociado al uso de nanomateriales como aditivos alimentarios también incluye experimentos *in vivo*, en los que se estudia la absorción, distribución, acumulación y eliminación del nanomaterial en animales. En este capítulo, se ha estudiado la acumulación de plata en distintos tejidos (músculo, hígado y riñón) y la eliminación en heces y purines de cerdos, analizando muestras provenientes de un experimento *in vivo*, donde se alimentó a cerdos con pienso suplementado con el aditivo Ag-caolín. Para ello, se determinó la concentración de plata total de tejidos, heces y purines mediante digestión ácida y análisis por ICP-MS. Los resultados obtenidos han mostrado que la plata procedente del pienso suplementado con Ag-caolín no se acumulaba en el músculo, pero sí lo hacía en el hígado y, en menor medida, en el riñón, aunque la mayor parte de la plata consumida se eliminaba a través de las heces. Debido al uso de los purines como abono, y la posible liberación de plata al medio ambiente, se evaluaron varios métodos de extracción para el fraccionamiento de las distintas especies de plata en heces, ya que no se dispuso de suficiente cantidad de purines para realizar los experimentos. Como reactivos de extracción se estudiaron: agua, hidróxido de tetrametilamonio (TMAH) y pirofosfato tetrasódico (TSPP) en combinación con amoniaco y sulfuro sódico. Como técnicas de detección de plata particulada y disuelta en los extractos se utilizaron SP-ICP-MS, HDC-ICP-MS y AF4-ICP-MS. Los resultados mostraron que la extracción con agua fue una buena opción para evaluar una posible liberación de plata al medio ambiente, obteniendo que menos de un 3% de la plata total presente en las heces se liberaría. La extracción con TMAH permitió la extracción simultánea de Ag(I) y partículas de plata, sin embargo, la eficiencia de extracción obtenida fue baja (42%), siendo un método adecuado para detectar partículas de plata. La extracción secuencial basada en TSPP permitió el fraccionamiento de plata en plata soluble y partículas de plata metálica, cloruro de plata y sulfuro de plata, obteniendo que

las fracciones más abundantes correspondieron a sulfuro de plata particulado (45%) y plata soluble (34%).

1. Introducción

El siguiente paso en la evaluación del riesgo asociado a nanomateriales utilizados en alimentación animal o humana, es la realización de experimentos *in vivo* en el que se estudien la absorción, distribución en tejidos, acumulación y eliminación de NPs provenientes del nanomaterial.

Como se ha mencionado en los capítulos anteriores, las AgNPs son ampliamente utilizadas debido a sus propiedades antibacterianas [1]. Esta propiedad las hace interesantes para ser utilizadas como aditivos en piensos, con el objetivo de sustituir los antibióticos, al poseer la misma propiedad antibacteriana [2]. Su bioacumulación en los órganos es un parámetro importante para conocer su posible toxicidad, tanto para el animal como para los consumidores de este. Antsiferova et al. [3] estudiaron la acumulación de AgNPs, provenientes de un suplemento alimentario comercial “Argovit S”, tras su administración oral en ratones en un periodo de uno a seis meses, observando que los órganos diana para las AgNPs, tras 180 días de exposición, eran los pulmones, el cerebro y el hígado, y que, cuando se dejaba de administrarlas, la plata se acababa eliminando del hígado. Gmoshinski et al. [4] también encontraron que el órgano diana de AgNPs era el hígado en ratas, obteniéndose una mayor concentración de plata acumulada en él, con tiempos de exposición de 90 días, a diferencia de órganos como el bazo o los riñones. Jiménez-Lamana et al. [5] administraron colargol, un nanomaterial base de AgNPs estabilizadas con caseína, a ratas por vía oral, observando la acumulación de plata en hígado y riñón y una elevada eliminación en heces. Resultados similares encontraron Abad-Álvaro et al. [6], que observaron que la acumulación de plata en cerdos alimentados con un aditivo base de plata se producía mayoritariamente en el hígado, y que disminuía al retirar el aditivo de la dieta, eliminándose la plata a través de las heces.

Conocer la forma en la que la plata se encuentra presente en las heces y purines también es importante para evaluar su toxicidad. En los últimos años se han desarrollado distintos métodos de preparación de muestra para digerir [7,8] muestras biológicas. Los métodos más utilizados son la extracción alcalina con TMAH [5,9–16] y la enzimática [17–22]. El

TMAH es una base fuerte, capaz de romper las matrices orgánicas y biológicas, al causar la escisión hidrolítica y metilación de los grupos éster, amida y éter, y de romper los puentes disulfuro de las proteínas [16]. Para evitar la precipitación de la Ag(I), debido a que el TMAH es una base fuerte, es necesario la adición de estabilizantes. Clark et al. [23] demostraron que el uso de TMAH en solitario, causaba la precipitación de Ag(I) en muestras de pescado. Vidmar et al. [22] obtuvieron resultados similares con muestras de placenta humana. Por ello, Clark et al. [23] propusieron el uso de CaCl_2 como estabilizante para evitar la precipitación de la Ag(I). Con el mismo objetivo, Jiménez-Lamana et al. [5] utilizaron cisteína para estabilizar Ag(I). Además, utilizaron Triton X-100 como surfactante para estabilizar AgNPs. El uso de surfactantes como estabilizantes también se ha estudiado en otros trabajos [16,24,25] para prevenir la pérdida de AgNPs. Generalmente, la extracción enzimática se lleva a cabo usando proteinasa K [25–27], pero esta enzima puede disolver parcialmente las AgNPs. Además, la presencia de residuos de la matriz puede llevar a la obtención de bajas recuperaciones de nanopartículas [21]. Al comparar la extracción alcalina y la enzimática [25,27], se obtuvieron mejores resultados con la extracción alcalina. Sung et al. [27] observaron que el TMAH ofrecía mejores recuperaciones (102%) que la extracción enzimática (74%) para AgNPs y AuNPs en muestras de hígados de pez cebra. Sin embargo, Loeschner et al. [19] no obtuvieron diferencias entre ambos métodos para la determinación de AuNPs en bazo de ratas y tampoco observaron efectos sobre la determinación del tamaño de partícula con las dos extracciones.

El TSPP se ha utilizado con frecuencia para la extracción y fraccionamiento de diferentes especies de metales en suelos, con especial atención a las formas particuladas [28–31]. Este reactivo mantiene la morfología de las partículas tras su extracción [31]. Hong et al. [30] desarrollaron un método de extracción secuencial para diferentes especies de plata (Ag(I), AgNPs, AgCl-NPs y Ag₂S-NPs) en suelos sobre los que se les había adicionado las distintas especies de plata. Este protocolo se basó en la utilización de TSPP y sulfuro de sodio (Na_2S) para separar las distintas especies. Mediante la utilización de TSPP, consiguieron separar Ag(I), AgNPs y AgCl-NPs. Para separar de esta fracción la parte correspondiente a Ag(I), posteriormente centrifugaron las muestras, quedándose con el

sobrenadante, mientras que la parte correspondiente a AgCl-NPs y Ag(I) la separaron mediante la adición de amoníaco. Por otra parte, la parte correspondiente a Ag₂S-NPs, la separaron mediante la adición de Na₂S. El Na₂S solubilizaría las partículas de Ag₂S por las especies de H₂S y HS⁻, que son las especies predominantes a pH 7, formando el complejo Ag₂S(H₂S)_x(HS)_y^{-y}. Esta especie se mantendría soluble y permitiría conocer la cantidad de Ag₂S-NPs total presente en las muestras. Con esta extracción se obtuvo información muy interesante, ya que proporcionó conocimiento sobre la naturaleza de las especies de plata y la proporción en la que se encuentran. Sin embargo, la utilización de amoníaco y Na₂S como extractantes, causó la disolución y acomplejamiento de AgCl y Ag₂S, lo que provoca una pérdida de información sobre las propiedades de estas partículas, como el tamaño o la morfología.

La extracción con agua se ha usado para estudiar las posibles transformaciones (por ejemplo, disolución, oxidación, agregación) de NPs durante el proceso de liberación en agua [32,33]. Torrent et al. [32] añadieron AgNPs a suelos para estudiar su adsorción. Posteriormente, sometieron los suelos a un proceso de lixiviación, obteniendo una baja movilización y biodisponibilidad de las AgNPs, encontrando que las partículas de tamaños más pequeño (40 nm) se oxidaban formando Ag(I). En una extracción con agua de suelos con heces de gusanos alimentados con NPs de cobalto, AgNPs y Ag(I), se obtuvo que la concentración de plata liberada era inferior al límite de detección de la técnica utilizada [34].

El objetivo del trabajo desarrollado en este capítulo es la evaluación de los riesgos derivados del uso del nanomaterial Ag-kaolin como aditivo alimentario en cerdos mediante el estudio de la acumulación de plata en tejidos y su eliminación en heces y purines. Además, se ha evaluado la eficiencia de tres estrategias de extracción diferentes, basadas en agua, TMAH y TSPP, para la extracción de distintas especies de plata en heces. El comportamiento de las especies de plata se ha estudiado mediante ICP-MS, para la determinación de los contenidos totales, y SP-ICP-MS, HDC-ICP-MS y AF4-ICP-MS para el análisis de especiación. Adicionalmente, se han utilizado técnicas de microscopía electrónica (TEM) para determinar el tamaño y composición de las partículas que se liberan.

2. Materiales y métodos

2.1. Instrumentación

Para la determinación de plata total se utilizó un espectrómetro ICP-MS Perkin Elmer NexION 2000. La optimización del equipo se describe en el Capítulo 2 (página 122), así como las condiciones de medida utilizadas en el modo convencional (tabla 2.1) y para el modo de detección de partículas individuales (tabla 2.2) (página 123).

El sistema cromatográfico de alta resolución que se utilizó fue un Waters 2796 (Waters Corporation, Milford, EE. UU.), cuya descripción se muestra en el Capítulo 1 (página 86), al igual que las condiciones experimentales empleadas en ICP-MS y HDC (tabla 1.1, página 87). La composición de la fase móvil utilizada fue 0.45 mM de SDS y 1 mM de penicilamina, filtrada doblemente y sonicada previamente.

Para el análisis por AF4-ICP-MS, se utilizó un sistema AF2000 (Postnova Analytics, Landsberg, Alemania). Se empleó un canal trapezoidal con una longitud de 14 cm y una anchura de 2 cm, equipado con un espaciador de 350 μm (Postnova, Landsberg, Alemania) y una membrana de poliétersulfona (PES), con un tamaño de corte de 5 kDa (Postnova, Landsberg, Alemania). El sistema de AF4 se acopló a un ICP-MS Perkin Elmer Sciex, (ELAN DCR-e, Perkin Elmer, Toronto, Canadá), cuya descripción se ha explicado anteriormente (Capítulo 1, página 86). La composición de la fase móvil fue de 0.01 M de NH_4NO_3 para el análisis de las proteínas y agua ultrapura para el resto de los experimentos. Ambas fases móviles se filtraron previamente. Las condiciones de medida utilizadas se muestran en la tabla 3.1.

Tabla 3.1. Condiciones de medida utilizadas en la medida con AF4-ICP-MS.

ICP-MS	
Potencia RF (W)	1100
Flujo del gas de argón	
Plasma (L min ⁻¹)	15
Auxiliar (L min ⁻¹)	1.2
Nebulizador (L min ⁻¹)	1.0
Parámetros de adquisición	
Barridos por lectura	20
Lecturas por réplica	
Con canal	400
FIAS-continuo	80
Tiempo de lectura (ms)	50
Modo de adquisición	<i>Peak hopping</i>
Isótopos monitorizados	¹⁰⁷ Ag, ¹⁰⁹ Ag
AF4	
Volumen de inyección (μL)	100
Fase móvil	NH ₄ NO ₃ y agua ultrapura

Para el análisis por TEM, se empleó un microscopio electrónico de transmisión Tecnai F30 (FEI Company, Eindhoven, Países Bajos) equipado con una fuente de electrones con un voltaje de entre 200 y 300 kV. Este sistema se acopló a un EDS. Para el procesado de imágenes, se utilizó el *software* Image-J, versión 1.53.

Para el procesamiento y representación de los datos adquiridos se utilizó el *software* Origin 9.6.5.169 (OriginLab Corporation, Northampton, MA, EE. UU.).

2.2. Reactivos

Las suspensiones de AgNPs utilizadas se prepararon a partir de suspensiones comerciales (NancoComposix, San Diego, EE. UU.) estabilizadas con citrato (2 mM), con diámetros nominales de 10, 20, 40 y 60 nm (diámetros certificados: 10.3 ± 2.1 , 18.6 ± 2.7 , 39 ± 5 , 59 ± 6 nm, respectivamente) y una concentración de 21 mg L⁻¹. También se utilizaron estándares de Ag(I) (994 ± 3 mg L⁻¹) y Au(III) (999 ± 4 mg L⁻¹) (Sigma-Aldrich, St. Louis, EE. UU.). El Rh, utilizado como patrón interno en ICP-MS, se preparó a partir de una disolución estándar con una concentración de 1002 ± 6 mg L⁻¹ (Sigma-Aldrich, St. Louis, EE. UU.). Las disoluciones se prepararon por pesada con una balanza analítica (GH-200, A&D, California, EE. UU.).

Para la preparación de la fase móvil utilizada en las separaciones por HDC (0.45 mM SDS y 1 mM penicilamina), se empleó DL-penicilamina (Sigma-Aldrich, Sta. Louis, EE. UU.) y SDS (Laboratorios Bio-Rad, Hercules, EE. UU.) El pH de la fase móvil fue de 7.5, que se ajustó con disoluciones de HNO₃ (69/70%, J.T. Baker, Phillipsburg, EE. UU.) y NaOH (Prolabo, Barcelona, España), con un pHmetro GLP 21 (Crison, España).

Los reactivos utilizados en los métodos de extracción fueron TMAH 25% (Alfa Aesar, Kandel, Alemania), TSPP (Sigma-Aldrich, Sta. Louis, EE. UU.) y Na₂S (Sigma-Aldrich, Sta. Louis, EE. UU.). También se emplearon cisteína (Sigma-Aldrich, Sta. Louis, EE. UU., BioUltra), Triton X-100 (Sigma-Aldrich, Sta. Louis, EE. UU.), CaCl₂ (Fisher Scientific, Loughborough, R.U.) y amoníaco (30%, J.T. Baker, Phillipsburg, EE. UU.).

Para la digestión ácida de las muestras de tejidos, se usó HNO₃ (69/70%, J.T. Baker, Phillipsburg, EE. UU.) y HCl (69/70%, J.T. Baker, Phillipsburg, EE. UU.). Para la validación de los resultados se utilizó un material certificado DOLT-4 (*Dogfish Liver Certified Reference Material for Trace Metals*, Consejo Nacional de Investigación de Canadá, Instituto para la Medida Nacional de Estándares, Canadá), cuya concentración en plata es $0.93 \pm 0.07 \text{ mg kg}^{-1}$.

Para llevar a cabo las calibraciones en tamaño en AF4, se utilizaron tres proteínas: pepsina de mucosa gástrica porcina (35 kDa) (Sigma-Aldrich, St. Louis, EE. UU.), albúmina de suero bovino (67 kDa) (Sigma-Aldrich, St. Louis, EE. UU.) y glucosa oxidasa de *Aspergillus niger* (160 kDa) (Sigma-Aldrich, St. Louis, EE. UU.).

Tanto para la preparación de las fases móviles como para las disoluciones y las diluciones se usó agua ultrapura (Milli-Q Advantage, Molsheim, Francia).

2.3. Estudio *in vivo*: obtención de muestras

Se utilizaron 60 lechones machos destetados a los 24 ± 3 días de edad y con un peso de $8.88 \pm 0.68 \text{ kg}$ que se distribuyeron en tres grupos, en función de la proporción de aditivo añadido en el pienso administrado: uno de los grupos recibió pienso sin aditivo (control), mientras que los otros dos grupos recibieron el pienso suplementado, uno con 20 mg de

Ag-kaolin kg^{-1} (Ag20) y el otro con 200 mg de Ag-kaolin kg^{-1} (Ag200). Cada grupo se alojó en cinco corrales diferentes. El cuidado y uso de los animales se realizó de acuerdo con la Política Española de Protección Animal RD53/2013, que cumple con la Directiva de la Unión Europea 2010/63/UE relativa a la protección de los animales con fines científicos.

Los primeros 14 días después del destete, los lechones se alimentaron con pienso de pre-inicio, los siguientes 21 días se alimentaron con pienso de inicio y las últimas 5 semanas se alimentaron con pienso libre de plata en todos los grupos. Se controló la mortalidad y la morbilidad todos los días, y los animales se pesaron semanalmente. Al final de la etapa de iniciación (35 días después del destete) y al final del experimento (30 días de pienso) se sacrificaron dos lechones por corral, y se tomaron muestras de heces de colon, hígado, músculo, riñones y purines para determinar el contenido de plata total.

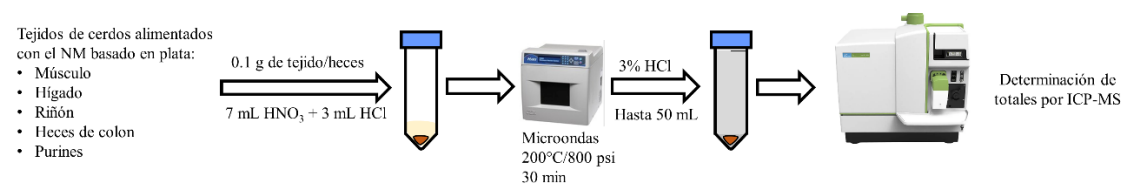
Las muestras se liofilizaron, y posteriormente, se molieron con un molino Restch MM400 (Restch, Düsseldorf, Alemania). Para el molido, se emplearon tarros de acero inoxidable con una bola de 25 mm. En los tarros se introdujeron 2 g de muestra liofilizada y cortada en trozos pequeños. Las condiciones de molido fueron 25 Hz de frecuencia durante 3 min.

2.4. Procedimientos

2.4.1. Determinación del contenido total de plata en tejidos, heces y purines

La digestión ácida de los tejidos (hígado, riñón y músculo), heces y purines de cerdos recolectados del ensayo *in vivo* se desarrolló siguiendo el protocolo descrito en Jiménez-Lamana et al. [5] (esquema 3.1). Para ello, se pesaron 0.1 g de tejido molido en tubos de teflón. A continuación, se añadieron 3 mL de HCl y 7 mL de HNO_3 . Las suspensiones se introdujeron en el microondas a 200°C y 800 psi durante 30 min. La potencia utilizada fue de 1600 W. Una vez las muestras se digirieron, la solución resultante se trasvasó a un tubo de polietileno y se llevó a 50 mL con una disolución de 3% de HCl (m/m) para su posterior análisis por ICP-MS. Para las diluciones necesarias de las muestras también se utilizó 3% de HCl (m/m). También se realizaron recuperaciones de material certificado, de blanco y de ataque.

Capítulo 3. Acumulación y eliminación de plata en cerdos alimentados con pienso suplementado con Ag-caolín. Estudio comparativo de métodos de extracción de especies de plata en heces

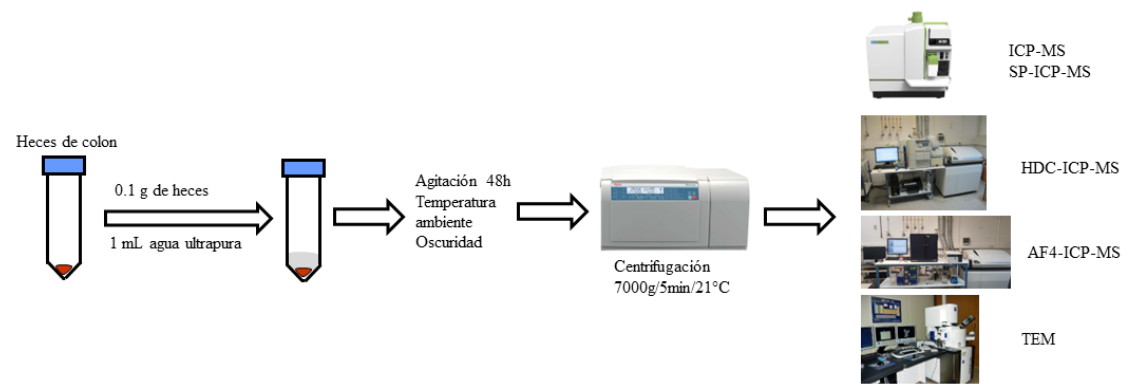


Esquema 3.1. Esquema de la digestión ácida de los tejidos y de las heces de cerdos.

Antes del análisis de las suspensiones resultantes tras la digestión ácida, se llevó a cabo una calibración con disoluciones de Ag(I) en 2% de HNO₃ (m/m) y 6% de HCl (m/m) con concentraciones entre 0 y 50 µg L⁻¹. Como patrón interno se utilizó una disolución de Rh de 10 µg L⁻¹ en 1% de HNO₃. Se utilizaron los parámetros instrumentales que se muestran en la tabla 2.1 del Capítulo 2 (página 122).

2.4.2. Extracción con agua

En el esquema 3.2 se muestra el protocolo utilizado para la lixiviación de heces con agua ultrapura. Se pesaron 0.1 g de heces molidas y se adicionó 1 mL de agua ultrapura, (relación sólido:líquido de 1:10) [35]. Las suspensiones se agitaron a 28 rpm durante 48 h a temperatura ambiente y en ausencia de luz. A continuación, las suspensiones se sonicaron durante 5 min y se dejaron 20 min reposar a temperatura ambiente. Posteriormente, las muestras se centrifugaron a 7000 g durante 5 min y 21°C para depositar en el fondo del tubo las partículas con un tamaño mayor de 1 µm. Se tomaron los sobrenadantes resultantes y se diluyeron con agua ultrapura.

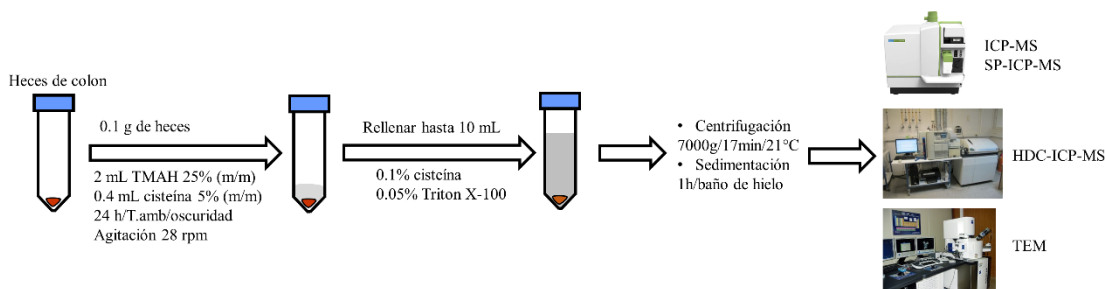


Esquema 3.2. Esquema del proceso de extracción de plata en heces con agua.

El contenido en plata total de los sobrenadantes se determinó mediante ICP-MS. Para la caracterización y cuantificación de las distintas especies de plata se utilizaron SP-ICP-MS, HDC-ICP-MS y AF4-ICP-MS. Además, para la detección de partículas también se utilizó TEM. Para este último, 20 μ L del sobrenadante de la extracción sin centrifugar se depositaron sobre una rejilla de cobre. Una vez la muestra se secó, se recubrió con carbono.

2.4.3. Extracción alcalina

Para llevar a cabo la extracción alcalina, se siguió el procedimiento descrito por Jiménez-Lamana et al. [5] (esquema 3.3). A 0.1 g de heces molidas se añadieron 2 mL de TMAH 25% (m/m) y 0.4 mL de cisteína 0.5% (m/m) en un tubo de polipropileno de 15 mL. Las suspensiones se dejaron agitando durante 24 h a 28 rpm, a temperatura ambiente y en oscuridad. Tras ello, las muestras se sonicaron y se llevaron a 10 mL con una disolución de cisteína de 0.1% (m/m) y Triton X-100 de 0.05% (m/m). Para investigar la posible pérdida de plata durante el proceso de centrifugado, se emplearon dos métodos de separación de la fase sólida tras la extracción alcalina. Por un lado, las muestras se centrifugaron a 7000 g durante 17 min a 21°C. Por otro lado, se realizó una sedimentación, dejando las muestras en un baño de hielo durante 1 h. Antes del análisis las muestras se diluyeron con agua ultrapura.



Esquema 3.3. Esquema del proceso de extracción con TMAH, cisteína y Triton X-100 de plata en heces.

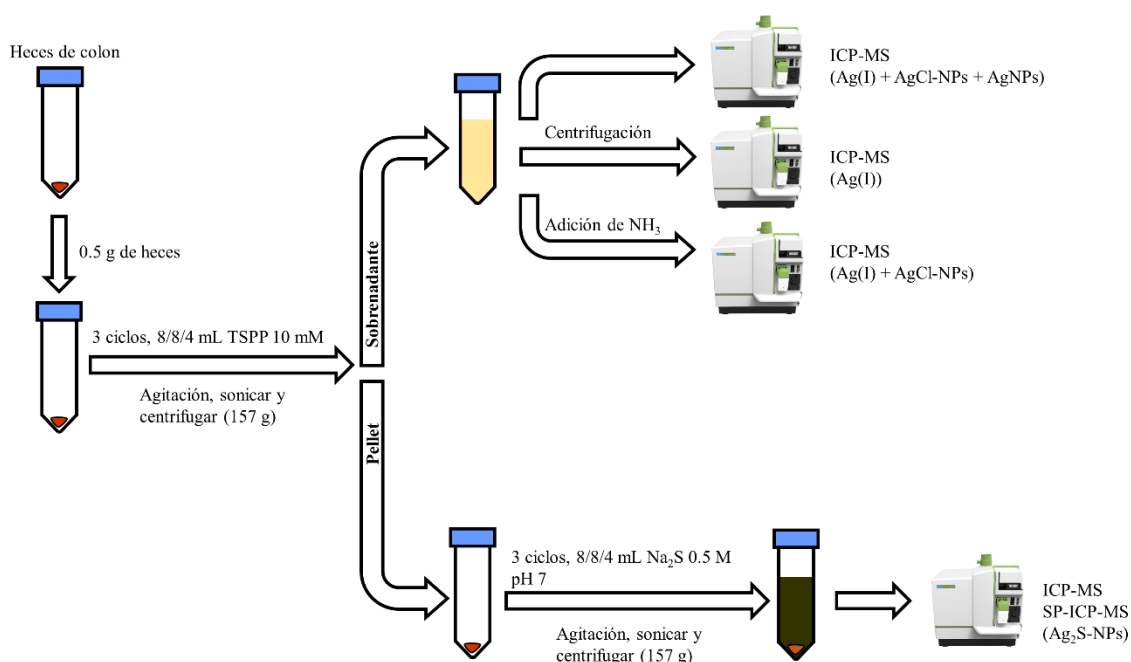
Para calcular el contenido en plata total de los sobrenadantes de la extracción, las muestras se midieron por ICP-MS. Para la detección, caracterización y cuantificación de las distintas especies de plata se utilizaron SP-ICP-MS y HDC-ICP-MS. Para detectar las partículas de plata, las muestras se midieron por TEM. Para este último, 20 μ L del

sobrenadante sin centrifugar se depositaron sobre una rejilla de oro y, una vez seca la muestra, se recubrió con carbono.

2.4.4. Extracción secuencial con TSPP

Para el proceso de extracción secuencial basada en el uso de TSPP, amoníaco y Na_2S , se empleó el protocolo desarrollado por Hong et al. [30] (esquema 3.4). A 0.5 g de heces molidas se le añadieron 8 mL de una disolución de 10 mM de TSPP, y se ajustó el pH con una disolución de 2 mM de NaOH. Las muestras se agitaron a 200 rpm y se dejaron en un baño de ultrasonidos durante 30 min. Posteriormente, se centrifugaron durante 10 min a 157 g. El sobrenadante resultante se separó del sólido, y sobre este último se repitió dos veces el mismo procedimiento, añadiendo 8 y 4 mL de la disolución de TSPP en cada caso. En total, el sobrenadante que se obtuvo tras los tres ciclos de extracción fue de aproximadamente 18 mL. Este sobrenadante se dividió en 3 alícuotas: una para determinación directa, que corresponde a la fracción de Ag(I), AgNPs y AgCl-NPs; la segunda, que se centrifugó, y que corresponder a la fracción de Ag(I), y la última, sobre la que se añadió NH_3 y se centrifugó, que corresponde a la fracción de Ag(I) y AgCl-NPs.

El sólido resultante tras la extracción con TSPP se lavó 3 veces con agua ultrapura y se le añadieron 8 mL de una disolución de 0.5 M de Na_2S a pH 7.0. Se siguió el mismo procedimiento descrito anteriormente para el TSPP, agitación, baño de ultrasonidos y centrifugación. El proceso se repitió dos veces más (con 8 y 4 mL de la disolución de Na_2S), obteniendo aproximadamente 18 mL de sobrenadante, que se midió por ICP-MS, tras dilución con agua ultrapura. Con este paso, se extrajo la fracción que corresponde a Ag_2S -NPs.



Esquema 3.4. Esquema del proceso de extracción secuencial basada en TSPP, amoníaco y Na_2S para el fraccionamiento de plata en heces.

Los sobrenadantes se midieron por ICP-MS, para obtener el contenido de plata total en cada fracción y por SP-ICP-MS para comprobar la presencia de partículas.

2.4.5. Cuantificación de plata total por ICP-MS

La plata total extraída en las tres extracciones se cuantificó por ICP-MS. Para ello, se utilizó una recta de calibrado de Ag(I) en 1% en HNO_3 , con concentraciones entre 0 y $15 \mu\text{g L}^{-1}$ y una disolución de $10 \mu\text{g L}^{-1}$ de Rh como patrón interno.

2.4.6. Caracterización y cuantificación de las distintas especies de plata por SP-ICP-MS

Para la cuantificación por SP-ICP-MS, se preparó una recta de Ag(I) en 1% en HNO_3 , con concentraciones entre 0 - $1 \mu\text{g L}^{-1}$ y una suspensión de AgNPs de 40 nm en agua ultrapura con una concentración de 1.50×10^5 partículas mL^{-1} . Previamente, se midió gravimétricamente el caudal de muestra y se calculó el rendimiento de nebulización (5.52%) mediante el procedimiento del tamaño descrito por Pace et al. [36], utilizando una recta de Au(III) en agua ultrapura con concentraciones entre 0 y $1 \mu\text{g L}^{-1}$, y con una suspensión de AuNPs de 47.8 nm y con una concentración en número de alrededor de

2.04×10^5 partículas mL^{-1} También se utilizó 5σ como criterio de discriminación entre la plata disuelta y las partículas.

2.4.7. Caracterización y cuantificación de las distintas especies de plata por HDC-ICP-MS

Para la caracterización y cuantificación de las distintas especies de plata por HDC-ICP-MS se prepararon una recta de calibrado de concentración de Ag(I) en 1 mM de penicilamina, con concentraciones entre 20 y 450 $\mu\text{g L}^{-1}$, y una recta de calibrado para los tamaños, con suspensiones de AgNPs de 10, 20, 40 y 60 nm ($250 \mu\text{g L}^{-1}$), que se ajustó a una curva logarítmica.

2.4.8. Caracterización y cuantificación de las distintas especies de plata por AF4-ICP-MS

Para las medidas por AF4-ICP-MS se utilizaron los programas de *crossflow* que se muestran en la tabla 3.2. Antes del análisis de las muestras, las condiciones de medida se optimizaron para conseguir la máxima sensibilidad para la plata, con una disolución de $20 \mu\text{g L}^{-1}$ de Ag(I) en 1% de HNO_3 preparada el mismo día de medida.

Tabla 3.2. Programas de *crossflow* utilizados en la determinación por AF4.

Programa	Etapas	Tiempo (min)		Crossflow (mL min^{-1})
Proteínas (medido por AF4)	Inyección/focusing	5	Flujo de inyección 0.20 mL min^{-1}	4
	Elución	20	Constante	3
		2	Caída lineal	De 3 a 0
		5	Constante	0
Nanopartículas (medido por AF4-ICP-MS)	Inyección/focusing	4	Flujo de inyección 0.20 mL min^{-1}	3
	Elución	5	Constante	3
		10	Caída lineal	De 3 a 0
		1	Constante	0

Para calcular el tamaño de partícula por AF4, se midieron tres suspensiones de proteínas de distintos tamaños (pepsina, albúmina y glucosa oxidasa), y se representó el logaritmo neperiano de sus tiempos de retención frente al logaritmo neperiano de su tamaño,

obteniendo una regresión lineal. El mismo procedimiento se siguió para disoluciones de estándares de AgNPs de 10, 20 y 40 nm con una concentración de $500 \mu\text{g L}^{-1}$, representando el logaritmo neperiano de sus tiempos de retención frente al logaritmo neperiano de sus tamaños.

Además, se llevó a cabo la ultrafiltración de las muestras y posterior medición por AF4-ICP-MS, utilizando cartuchos con una membrana de 100 kDa.

3. Resultados y discusión

3.1. Determinación del contenido de plata total en tejidos, heces y purines

El contenido de plata total de las heces, purines y tejidos (músculo, hígado y riñón) resultantes del ensayo *in vivo*, se cuantificó mediante digestión ácida (esquema 3.1 en la sección 2.4.1 de Procedimientos) y posterior análisis por ICP-MS.

Los resultados se validaron utilizando el material certificado DOLT-4 (hígado de cazón) y recuperaciones sobre blancos de procedimiento y muestras control. Se realizaron también recuperaciones de método, mediante la adición de Ag(I) a una muestra control, y del blanco. Se observa que las recuperaciones en los tres casos son próximas al 100%.

La concentración de plata total en los distintos tejidos se muestra en la tabla 3.3. Se observó que la plata se acumuló principalmente en hígado, en menor media en riñón, pero no en el músculo. Esto es esperable, puesto que el hígado y riñón son órganos diana de la plata [11,37,38] y el periodo de alimentación sin aditivo a base de plata contribuye a la eliminación de ésta del músculo. Ryan et al. [39] también encontraron acumulación de plata en cerebro, bazo e intestino, además de hígado. A la vista de los resultados, se observa que la mayor parte de la plata suministrada a los cerdos se eliminó en las heces y en purines, en las que se encontró una alta concentración de plata, como también se observó en la bibliografía [6,40]. Para las heces se obtuvo una concentración de 155 mg kg^{-1} para los cerdos alimentados con pienso suplementado con 20 mg kg^{-1} de plata (Ag20) y 1026 mg kg^{-1} con 200 mg kg^{-1} de plata (Ag200). En los purines se encontró una concentración de 109 mg kg^{-1} , en materia seca.

Tabla 3.3. Concentración de plata total en tejidos, heces y purines de cerdos. Media $\pm$ desviación estándar ($n=47$).

Muestra	Concentración de plata total (mg kg ⁻¹)		
	Control	Ag20	Ag200
Músculo	<LOQ	<LOQ	<LOQ
Riñón	<LOQ	<LOQ	0.38 $\pm$ 0.18
Hígado	<LOQ	0.60 $\pm$ 0.25	4.70 $\pm$ 2.10
Heces	0.70 $\pm$ 0.26	155 $\pm$ 30	1026 $\pm$ 178
Purines	5.18 $\pm$ 0.12*	109 $\pm$ 6*	-
Recuperación de método	96.0 $\pm$ 4.9 %		
Recuperación de blanco	96.3 $\pm$ 5.7 %		
Recuperación de material certificado	101 $\pm$ 1 %		

*En materia seca
LOQ: 0.119 mg kg⁻¹

En un principio, la incorporación del aditivo Ag-caolín en la alimentación de los cerdos en las condiciones del experimento *in vivo* no supuso la acumulación de plata en músculo, incluso suministrando cantidades diez veces superiores a las recomendadas, lo cual no afecta a la seguridad de su consumo. Sin embargo, la eliminación de la plata ingerida a través de las heces y su incorporación a los purines supone un riesgo potencial desde el punto de vista medioambiental, ya que son habitualmente utilizados como fertilizantes en agricultura. En función de la forma química en la que se encuentre la plata y su posible movilización, el impacto puede ser más o menos significativo. Aunque al finalizar el estudio *in vivo* se tomaron muestras de los purines almacenados en la granja, los cuales se liofilizaron para posteriores estudios, las cantidades obtenidas no fueron suficientes, por lo que se optó por utilizar las heces extraídas del colon de los animales como mejor aproximación a los desechos animales que se incorporarán al medio ambiente. Por este motivo, el resto de los estudios recogidos en este capítulo se centran en heces.

3.2. Métodos de extracción de especies de plata en heces: estudio comparativo

Se evaluaron tres métodos de extracción de plata: lixiviación con agua, extracción alcalina con TMAH y extracción secuencial basada en TSPP.

3.2.1. Extracción con agua

La extracción con agua simula la lixiviación/liberación al medio ambiente de sustancias en materiales de desecho [35]. La tabla 3.4 muestra el porcentaje de plata total que se liberó en la extracción con agua frente a la concentración total que se obtuvo por digestión ácida (tabla 3.3) por ICP-MS y SP-ICP-MS. En la misma tabla se muestra el porcentaje de Ag(I) y partículas de plata obtenido por SP-ICP-MS.

Tabla 3.4. Porcentaje de plata total liberada frente a la plata total presente en heces tras la extracción con agua medido por ICP-MS y SP-ICP-MS. Media $\pm$ desviación estándar ($n=9$).

Técnica	Plata total liberada (%)	Ag(I) liberada (%)	Partículas de plata liberadas (%)
ICP-MS	2.55 ± 0.90	-	-
SP-ICP-MS	2.36 ± 1.06	2.25 ± 0.99	0.110 ± 0.085

El porcentaje de plata total liberada durante la extracción en agua por ICP-MS fue de $2.55 \pm 0.90\%$ ($n=9$), que corresponde a una concentración de plata de $3.75 \pm 1.13 \text{ mg kg}^{-1}$ ($n=5$) para las muestras de Ag20 y $20.3 \pm 4.3 \text{ mg kg}^{-1}$ ($n=4$) para las muestras de Ag200. Este porcentaje se mantiene constante independientemente de la cantidad de plata presente en las heces. Valores similares para la extracción en agua se encontraron en la bibliografía. Torrent et al. [32] obtuvieron valores de recuperación inferiores al 8% para Ag(I).

El pequeño porcentaje de plata liberada indica que el impacto derivado de la liberación en las heces al medioambiente sería bajo, en el caso de que se utilizaran como fertilizante.

3.2.1.1. Determinación de las distintas especies de plata en la extracción con agua por SP-ICP-MS

El porcentaje de liberación que se obtuvo por SP-ICP-MS fue $2.25 \pm 0.99\%$ ($n=9$) para Ag(I) y $0.110 \pm 0.085\%$ ($n=9$) para partículas de plata (tabla 3.4). Se observó la aparición de pocas partículas (distribuciones de masa y diámetro equivalente en figura 3.1), entre un 1-5% de la plata liberada lo hacía en forma nanoparticulada. La masa de plata por partícula más frecuente fue menor a 1000 ag (971 ag) (figura 3.1a), equivalente a partículas de plata con un diámetro de 34 ± 2 nm (figura 3.1b). En posteriores análisis por HDC-ICP-MS (sección 3.2.1.2) y TEM (sección 3.2.1.3), se observó además la presencia de partículas de tamaños más grandes, desde los 100 nm hasta 1 μm .

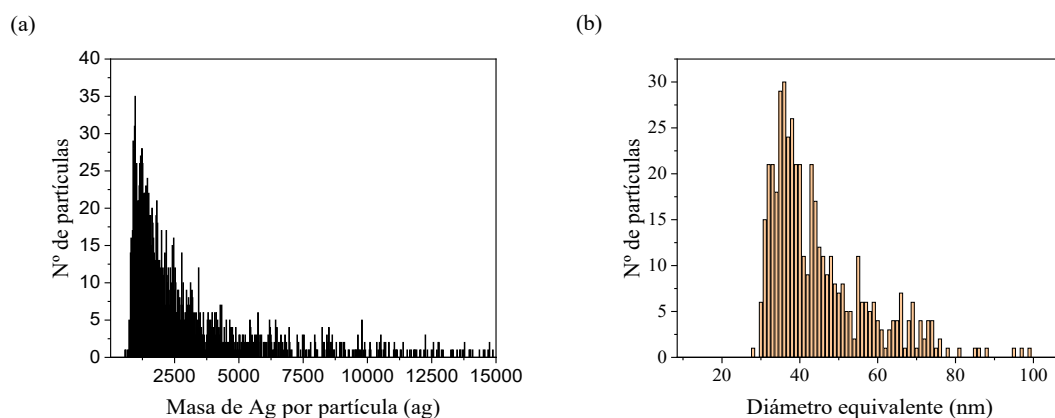


Figura 3.1. Distribuciones obtenidas por SP-ICP-MS para las partículas que contienen plata para el extracto acuoso de una muestra. (a) Distribución de frecuencia de masa de plata por partícula (ag). (b) Distribución de diámetro equivalente (nm).

Como se ha explicado en la Introducción (página 45), la detección de nanopartículas por SP-ICP-MS se ve limitada por los límites de detección en tamaño/masa de elemento por partícula, que se ve afectado por la concentración de la especie disuelta que en este caso es la forma mayoritaria liberada. Para reducir el efecto del disuelto sobre las distribuciones obtenidas, se realizaron una serie de diluciones secuenciales, con el objetivo de reducir la línea base hasta valores del blanco de agua ultrapura [42]. Los resultados se muestran en la tabla 3.5. Se observó que el tamaño equivalente más frecuente obtenido disminuía al aumentar la dilución realizada. De la misma forma, en

las distribuciones (figura 3.2), se observó que éstas se desplazaban a la izquierda (a tamaños más pequeños) a medida que la dilución era mayor y, por tanto, la concentración del disuelto disminuía. Esto nos demuestra que inicialmente no estábamos detectando la parte de la distribución correspondiente a partículas con contenidos menores de plata. Esto tiene sentido, ya que a medida que la concentración de Ag(I) disminuye, el LOD (calculado según la fórmula A.2 en el apéndice 1) es menor, permitiendo detectar partículas con menores contenidos de plata. Por contra, al aumentar la dilución, la concentración de partículas disminuye, reduciéndose el número de eventos medidos, lo que afecta a la cuantificación de su concentración. Aun así, llegar a estos valores no garantiza que no haya partículas con menores contenidos de plata, por lo que estos contenidos/diámetros equivalentes podrían estar sobrestimados y la proporción de plata particulada subestimada.

Tabla 3.5. *Tamaño, $LOD_{tamaño}$, concentración y número de eventos para las distintas diluciones de la extracción en agua para una muestra de Ag20 y Ag200 por SP-ICP-MS. Media $\pm$ desviación estándar ($n=3$).*

Muestra	Dilución	Tamaño equivalente más frecuente (nm)	$LOD_{tamaño}$ (nm)	Concentración de Ag(I) ($\mu\text{g L}^{-1}$)	Nº de eventos obtenidos
Ag20	1:500	45 ± 1	35.4	0.35 ± 0.01	328 ± 15
	1:625	41 ± 0	31.8	0.19 ± 0.01	258 ± 29
	1:1000	37 ± 2	24.9	0.05 ± 0.01	82 ± 3
Ag200	1:250	37 ± 1	25.3	0.69 ± 0.01	1554 ± 8
	1:500	32 ± 0	21.9	0.30 ± 0.01	948 ± 4
	1:1000	26 ± 1	16.5	0.07 ± 0.01	676 ± 14

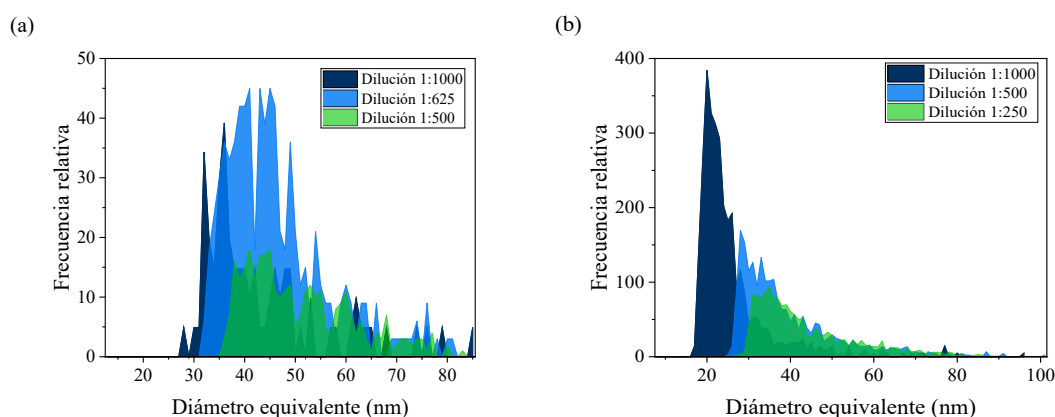


Figura 3.2. *Comparativa de las distribuciones de tamaño obtenidas por SP-ICP-MS para distintas diluciones de los extractos acuosos de muestras de heces. (a) Ag20 (b) Ag200.*

Para estudiar el comportamiento de las distintas especies de plata se realizaron adiciones de Ag(I) y AgNPs (40 nm) a una muestra control y se llevó a cabo el mismo proceso de lixiviación en agua. Para la adición de Ag(I), no se detectaron partículas, lo que indica que no se produjo precipitación o reducción de plata durante la extracción de forma significativa. Sin embargo, en el caso de la adición de AgNPs, cuya distribución de tamaño se muestra en la figura 3.3, el tamaño más frecuente de partícula que se obtuvo (28.9 ± 2.5 nm) era inferior al original (40 nm), además de un aumento de la línea base. Esto implicaría que las partículas se oxidaban durante el proceso de extracción, como se también se ha observado en otros trabajos [32,41].

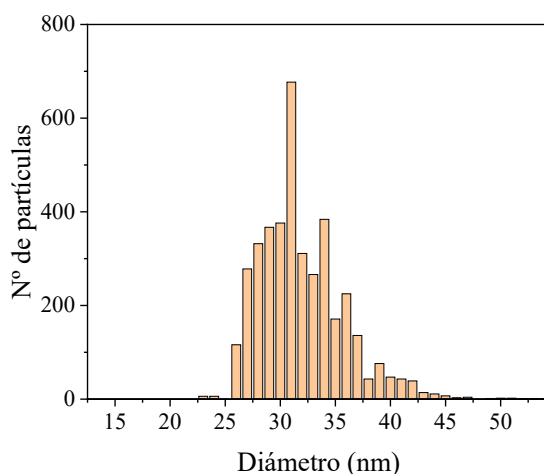


Figura 3.3. Distribución de tamaño obtenida por SP-ICP-MS para el extracto acuoso de una adición de AgNPs de 40 nm sobre una muestra control.

3.2.1.2. Determinación de las distintas especies de plata en la extracción con agua por HDC-ICP-MS

El lixiviado en agua se analizó también por HDC-ICP-MS. En la figura 3.4 en negro se muestra el cromatograma obtenido para la extracción en agua ultrapura de una muestra de heces Ag200. Se observaron dos picos, uno a 9.00 min, que corresponde a Ag(I) (el tiempo de retención del estándar de Ag(I) en penicilamina es de 8.86 min), y otro a 7.73 min, que corresponde a partículas que contienen plata con tamaños mayores a 300 nm. Este tamaño corresponde al límite de tamaño dado por el fabricante de la columna de HDC como tamaño máximo de separación.

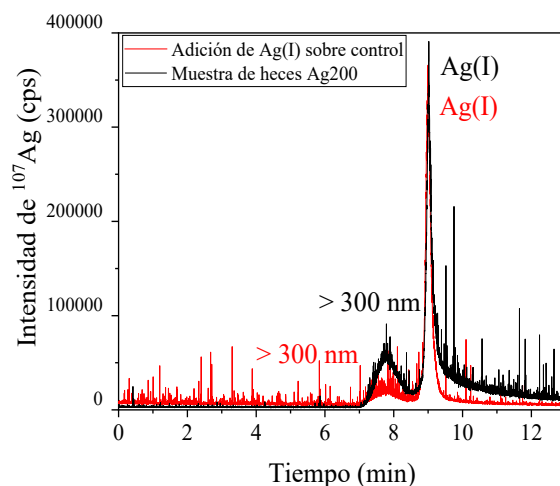


Figura 3.4. Cromatogramas obtenidos por HDC-ICP-MS para los extractos acuosos de una muestra de heces y una adición de Ag(I) sobre muestra control de heces.

Este cromatograma es similar al obtenido al realizar la lixiviación de una adición de Ag(I) sobre una muestra control de heces (figura 3.4 en rojo). De igual forma, en este cromatograma aparecen dos picos, uno a 9.00 min, que corresponde a Ag(I) y otro pico a 7.73 min, que correspondería a plata unida a partículas con tamaños superiores a 300 nm, probablemente a partículas de caolín. Este cromatograma confirma la presencia de plata unida a partículas grandes presentes en los extractos.

Por el contrario, en el caso del lixiviado de una muestra control con adición de AgNPs de 40 nm (figura 3.5), se observa la aparición de dos picos, uno con un tiempo de retención de 9.03 min, que corresponde a Ag(I) y otro a 8.04 min, que correspondería a AgNPs, cuyo tamaño no se pudo cuantificar correctamente debido al desplazamiento de los picos por efecto de la matriz de heces. Esto confirmaría la oxidación de las AgNPs durante el proceso de extracción, tal y como se obtuvo también por SP-ICP-MS (figura 3.3).

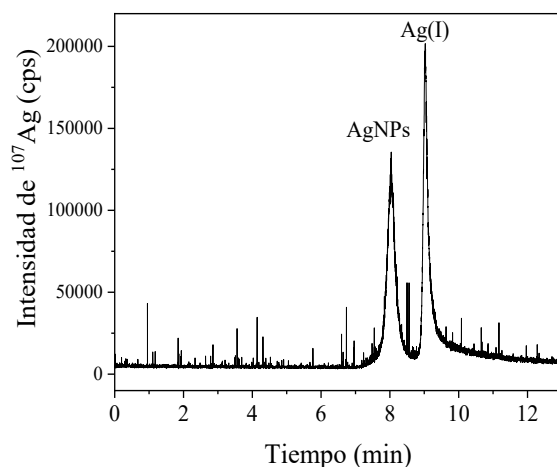


Figura 3.5. Comparativa de cromatograma obtenido por HDC-ICP-MS para el extracto acuoso de una muestra control de heces con adición de AgNPs (40 nm).

3.2.1.3. Detección de partículas de plata en la extracción con agua por TEM

Los sobrenadantes resultantes de la extracción con agua de las muestras se analizaron por TEM. En la figura 3.6 se muestra una imagen de las partículas obtenidas y su correspondiente espectro de EDS. Se observaron partículas y agregados con tamaños entre 100 nm y 1 μ m, lo que confirmaría los resultados obtenidos por HDC-ICP-MS. Por EDS, se observó la aparición de S, lo que indicaría la posible presencia de Ag₂S, mientras que Si y Al indicarían la presencia de partículas de caolín asociadas a la plata.

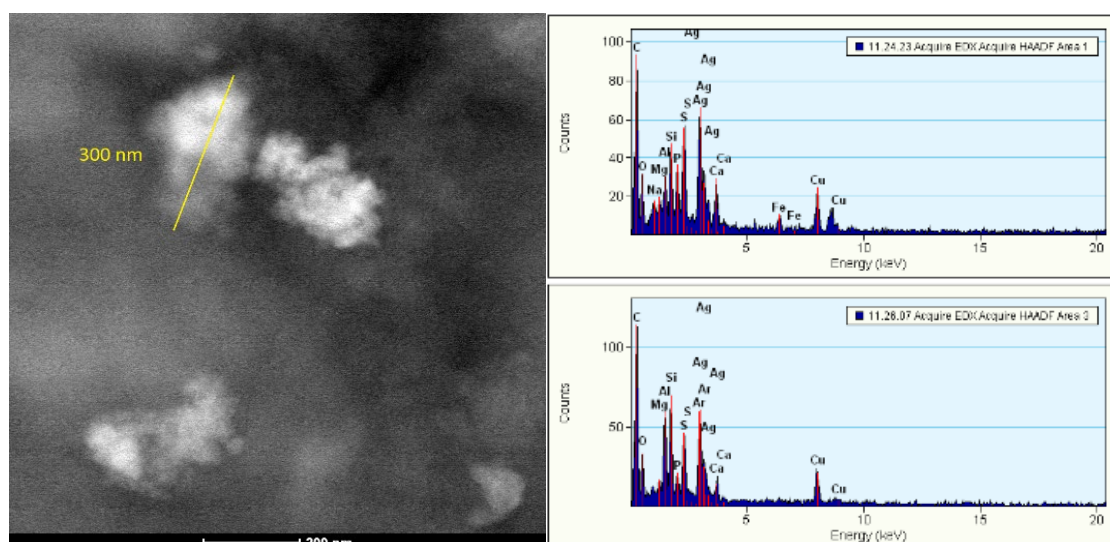


Figura 3.6. Imagen obtenida por TEM y espectro de EDS de las partículas que contienen plata en la extracción en agua de heces.

3.2.1.4. Determinación de las distintas especies de plata en la extracción de heces con agua por AF4-ICP-MS

Con objeto de profundizar más en la naturaleza de las especies de plata liberadas en la extracción con agua ultrapura y más teniendo en cuenta que tanto por HDC-ICP-MS y TEM se obtuvieron tamaños de partícula grandes, los sobrenadantes se analizaron también por AF4-ICP-MS.

Inicialmente, se llevaron a cabo las medidas con el programa optimizado para la separación de macromoléculas, cuya descripción aparece en la tabla 3.2 del apartado de procedimientos (sección 2.4.8). Los fractogramas obtenidos para el sobrenadante de la lixiviación en agua de heces Ag200 y las tres proteínas medidas se muestran en la figura 3.7. En la figura 3.7a se muestra una visión total de los fractogramas obtenidos, mientras que en la figura 3.7b se muestran los fractogramas ampliados. En la muestra de heces se obtuvieron dos picos, uno a 7.55 min y otro más grande a 27.9 min. El tiempo de retención de 7.55 min se interpoló en la recta del logaritmo neperiano de tiempos de retención frente al logaritmo neperiano de tamaño, obteniendo un tamaño de 25.4 kDa, que equivale a un tamaño menor de 5 nm, aproximadamente. El tamaño al que corresponde el segundo pico no fue posible caracterizarlo con este programa puesto que a ese tiempo se deja de aplicar un *crossflow* y no hay retención en la membrana. Este pico correspondería a partículas de

tamaños grandes que no permanecen en el canal y que se eluyen cuando se deja de aplicar el *crossflow*.

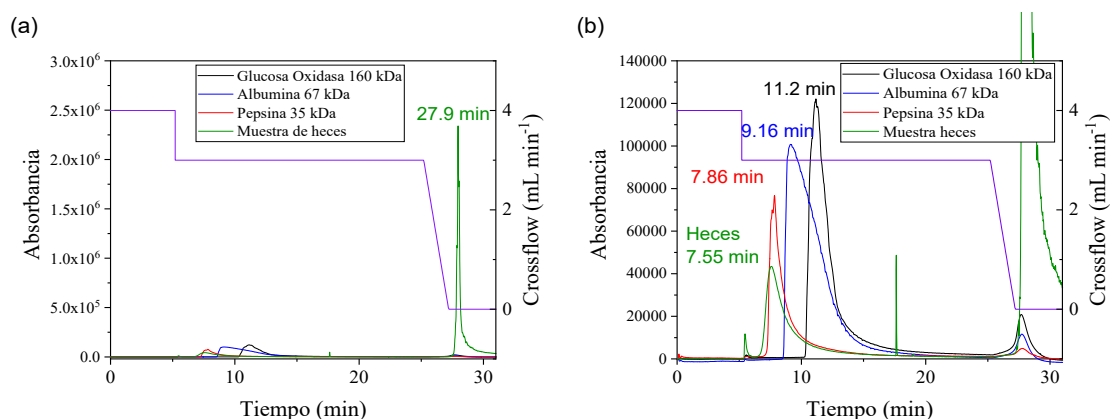


Figura 3.7. Fractogramas obtenidos para la lixiviación de una muestra Ag200 y para distintas proteínas. (a) Fractograma completo. (b) Fractograma ampliado en los picos necesarios para la calibración.

A la vista de los anteriores resultados, se utilizó un segundo programa diseñado para la separación de partículas, descrito en la tabla 3.2 en el apartado de procedimientos (sección 2.4.8), con el objetivo de determinar el tamaño del segundo pico que aparece en la muestra de heces. Inicialmente, se midieron suspensiones de estándares de AgNPs de 10, 20 y 40 nm ($500 \mu\text{g L}^{-1}$) (figura 3.8) para obtener la recta de calibrado y, posteriormente, las muestras de heces.

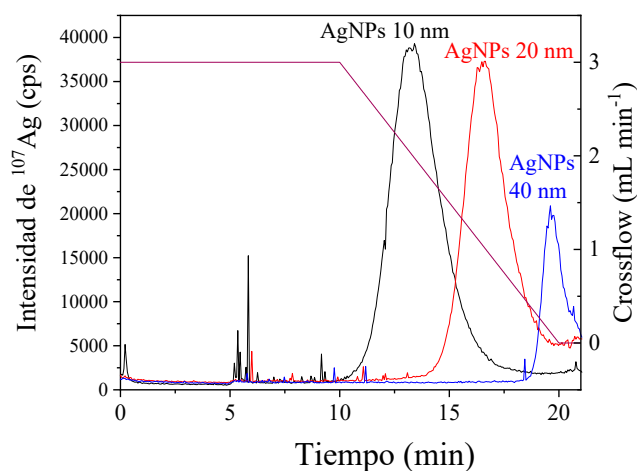


Figura 3.8. Fractogramas obtenidos por AF4-ICP-MS para disoluciones estándar de AgNPs.

El fractograma obtenido para la muestra de heces se muestra en la figura 3.9. Los resultados que se obtuvieron están en consonancia con los obtenidos por HDC-ICP-MS (figura 3.4) y TEM (figura 3.6). En el fractograma (figura 3.9) apareció un pequeño pico a 6.40 min, que corresponde a formas de plata asociada a macromoléculas (de entre 3 y 34 kDa), y otro pico más grande que corresponde a partículas mayores de 10 nm. Con el objetivo de mejorar la resolución, se llevaron a cabo sucesivas diluciones de la muestra, aunque no se consiguió mejorar la separación, al observarse un desplazamiento del pico correspondiente a las partículas de mayor tamaño a tiempos de retención menores.

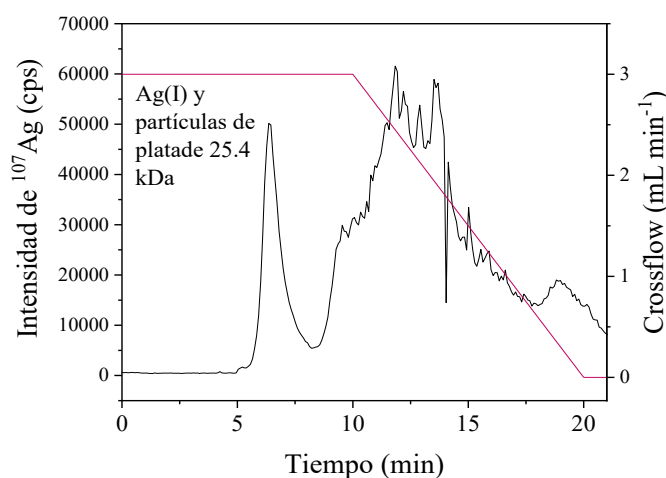


Figura 3.9. Fractograma obtenido por AF4-ICP-MS para la extracción en agua del extracto de una muestra sin dilución.

Con el objeto de poder confirmar que el primer pico obtenido, con un tiempo de retención en torno a 6 min, corresponde a plata disuelta unida a materia orgánica de bajo peso molecular, se llevó a cabo la ultrafiltración de los extractos. Para esta ultrafiltración se utilizaron cartuchos con una membrana de 100 kDa. En la figura 3.10 se muestran los fractogramas obtenidos. Como se puede observar, tras la ultrafiltración (en rojo) apareció un solo pico, correspondiente a plata disuelta asociada a macromoléculas de pequeño tamaño. No apareció el pico correspondiente a especies de plata de gran tamaño (tiempo de retención superior a los 10 min). Esto confirmaría que el segundo pico obtenido en el fractograma que se muestra en la figura 3.9, corresponde a especies de plata de tamaños superiores a 10 nm, como ya se vio por HDC-ICP-MS (figura 3.4) y por TEM (figura 3.6).

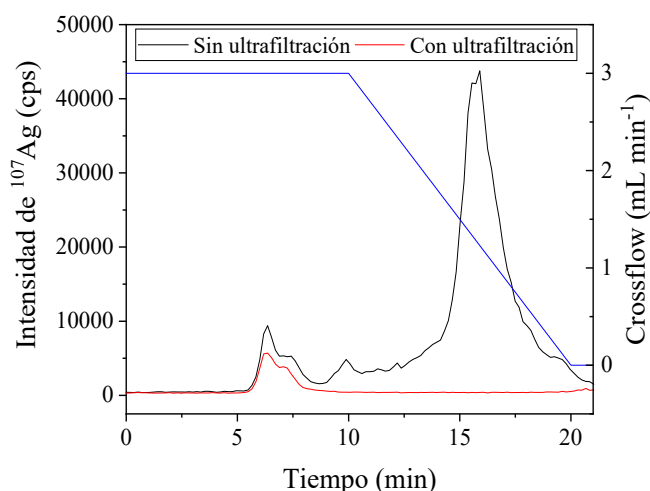


Figura 3.10. Comparativa de los fractogramas obtenidos de los lixiviados en agua con y sin ultrafiltración por AF4-ICP-MS.

3.2.2. Extracción alcalina

La extracción alcalina con TMAH se ha utilizado en la bibliografía para extraer AgNPs de diversos tejidos biológicos [5,9–12,16]. El TMAH es capaz de digerir la materia orgánica y las matrices biológicas, debido a su habilidad para romper los puentes disulfuro de las proteínas [10,16] sin provocar cambios en las nanopartículas.

Utilizando el método descrito por Jiménez-Lamana et al. [5], se llevó a cabo la extracción con TMAH, cisteína y Triton X-100, y los extractos obtenidos se analizaron por ICP-MS. Los resultados se muestran en la tabla 3.6. Se extrajo en torno a un 18% para ambos tipos de muestras. Las condiciones de centrifugación utilizadas en el artículo citado [5] fueron 3000 rpm durante 15 min, sin embargo, en este estudio se observó que, bajo estas condiciones, parte de la plata se quedaba retenida en el sólido. Por ello, se compararon como métodos de separación, la centrifugación y la sedimentación (1 h) y se estudió la posible pérdida de plata durante el proceso. Utilizando sedimentación se obtuvo un porcentaje de liberación de $42 \pm 11\%$ ($n=6$), por lo que se confirmó la pérdida de plata durante el proceso de centrifugación. Estos resultados coinciden con lo obtenido en la bibliografía [28,31,43,44], donde se reportó la pérdida de AgNPs en los sobrenadantes

tras la centrifugación. Por ello, se eligió la sedimentación en baño de hielo como método de separación en la extracción alcalina de heces.

Tabla 3.6. Cuantificación de plata total obtenida por ICP-MS para la extracción alcalina. Media $\pm$ desviación estándar.

Muestra	Concentración de plata total liberada (mg kg ⁻¹)*		Plata total liberada vs plata total (%)**	
	Centrifugación	Sedimentación	Centrifugación	Sedimentación
Ag20	21.8 $\pm$ 3.8	74.3 $\pm$ 10.2	18.0 $\pm$ 4.8	42 $\pm$ 11
Ag200	218 $\pm$ 28	401 $\pm$ 56		

* n=3

** n=6

Con objeto de estudiar el bajo porcentaje de extracción obtenido, se aplicó la extracción alcalina sobre muestras control a las que se había adicionada Ag(I) y AgNPs de 40 nm, obteniéndose valores de recuperación entre 40-50% similares a los obtenidos para las muestras originales. Se puede deducir que la matriz de heces retiene parte de la plata adicionada, lo cual justifica su extracción incompleta en las muestras originales. Debido a que el tratamiento con TMAH no consigue la digestión completa de la matriz de heces, la causa de esta baja eficiencia de extracción estaría en el sólido remanente no digerido, que actuaría como una “trampa” para la plata, evitando su extracción completa [45]. De hecho, cuando se llevó a cabo la extracción alcalina de Ag(I) y AgNPs (40 nm) sin matriz, se obtuvieron valores de recuperación mayores del 90% (tabla 3.7). Sin embargo, cuando se realizó la extracción sobre muestras adicionadas, la recuperación disminuyó hasta un 40-50%, lo que confirmó el efecto de la matriz en la extracción de la plata.

Tabla 3.7. Recuperaciones obtenidas por ICP-MS tras la extracción con TMAH para estándares de Ag(I) y AgNPs y adiciones sobre control Media $\pm$ desviación estándar (n=3).

Especie	Recuperación de plata (%)
Ag(I)	92.6 $\pm$ 9.5
AgNPs de 40 nm	95.3 $\pm$ 0.9
Adición de Ag(I)	41.3 $\pm$ 7.2
Adición de AgNPs de 40 nm	54.5 $\pm$ 1.2

3.2.2.1. Determinación de las distintas especies de plata en la extracción alcalina por SP-ICP-MS

Los extractos de TMAH se analizaron por SP-ICP-MS. Inicialmente, se estudió el efecto de TMAH, cisteína y Triton X-100 sobre la recta de calibrado y el rendimiento de nebulización. Los resultados se muestran en la tabla 3.8. Al aplicar un test t de *Student* sobre las pendientes de las rectas de calibrado no se observaron diferencias significativas (la t calculada fue de 1.92 frente a la t de referencia que es de 2.92, con un nivel de confianza del 90%). Sin embargo, el rendimiento de nebulización sí se vio afectado por el medio, en concreto, aumentaba con el uso del TMAH, obteniendo un valor de t superior al de referencia [12,22], por lo que se utilizó TMAH, cisteína y Triton para la preparación de las disoluciones.

Tabla 3.8. Rectas de calibrado y rendimiento de nebulización obtenidos para los diferentes medios por SP-ICP-MS. $t_{ref} = 2.92$.

Medio	Pendiente	Desviación pendiente	t_{cal}	Rendimiento nebulización (%)	Desviación rendimiento nebulización	t_{cal}
1% de HNO ₃	11.6	0.38	-	7.22	0.03	-
TMAH, cisteína y Triton X-100	12.4	0.1	1.92	8.63	0.10	13

La concentración de las distintas especies de plata obtenidas por SP-ICP-MS en la extracción con TMAH de heces se muestra en la tabla 3.9. La mayor parte de la plata extraída de las heces estaba en forma de Ag(I), entre el 90-99%, mientras que, entre el 1 y el 10% restante estaba en forma de plata particulada. En la figura 3.11a se observa la distribución en masa que se obtiene en la extracción alcalina por SP-ICP-MS. La masa de plata por partícula más frecuente obtenida estaba en torno a 360 ag, lo que corresponde a un tamaño de 40 nm, aproximadamente (distribución en tamaño en figura 3.11b).

Tabla 3.9. Resultados obtenidos en la extracción alcalina por SP-ICP-MS. Media $\pm$ desviación estándar ($n=3$).

Muestra	Diám. más frecuen. (nm)	Concen. partículas de plata extraídas (mg kg ⁻¹)	Concen. Ag(I) extraída (mg kg ⁻¹)	Concen. de plata total extraída (mg kg ⁻¹)	Ag(I) extraída vs. Plata total extraída (%)	Partículas de plata extraídas vs. plata total extraída (%)
Ag20 826	31 $\pm$ 1	0.045 $\pm$ 0.002	27.1 $\pm$ 0.1	27.2 $\pm$ 0.1	99.8 $\pm$ 0.1	0.17 $\pm$ 0.06
Ag20 825	41 $\pm$ 1	0.031 $\pm$ 0.002	41.5 $\pm$ 0.1	41.5 $\pm$ 0.1	99.9 $\pm$ 0.1	0.07 $\pm$ 0.01
Ag200 865	36 $\pm$ 2	6.03 $\pm$ 2.26	91.2 $\pm$ 13.2	97.2 $\pm$ 11.2	93.5 $\pm$ 3.0	6.47 $\pm$ 3.01
Ag200 859	42 $\pm$ 5	10.1 $\pm$ 4.3	68.6 $\pm$ 3.4	78.7 $\pm$ 1.9	87.2 $\pm$ 5.3	12.8 $\pm$ 5.3

Diám. más frecuen.: diámetro equivalente más frecuente

Concen.: concentración

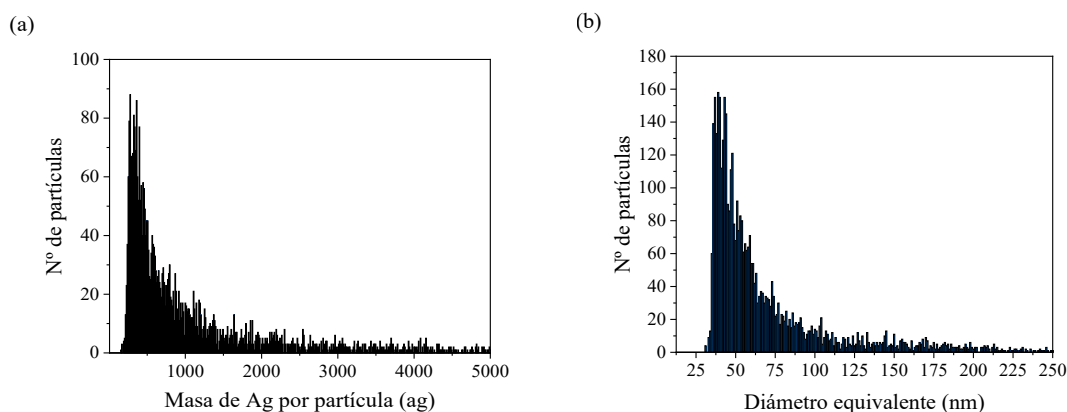


Figura 3.11. Distribuciones obtenidas por SP-ICP-MS para la extracción alcalina con TMAH, cisteína y Triton X-100 de una muestra de Ag200. (a) Distribución en masa por partícula de plata (b) Distribución de diámetro equivalente.

Para eliminar el posible efecto de la plata disuelta en las distribuciones resultantes en la extracción, se realizaron sucesivas diluciones para conseguir obtener una línea base similar a la obtenida en el blanco de agua ultrapura. Los resultados obtenidos se muestran en la tabla 3.10. El tamaño más frecuente obtenido disminuía al aumentar la dilución. Esto se debe a que, como se ha dicho anteriormente en la Introducción (página 45), al disminuir la cantidad de disuelto, también disminuye el LOD (calculado siguiendo la fórmula A.2 mostrada en el apéndice 1). Esto demuestra que la distribución detectada no es completa y que parte de ésta, la que corresponde a las partículas más pequeñas, no se detecta. Sin embargo, al disminuir la concentración del disuelto, sí se observó la parte de la distribución correspondiente a las partículas más pequeñas (figura 3.12). Pese a ello, al aumentar la dilución, se reduce el número de eventos medidos ya que disminuye la concentración de partículas, lo que afecta a la cuantificación de su concentración. Aun

así, llegar a estos valores no garantiza la ausencia de partículas con contenidos de plata menores, por lo que la proporción de plata particulada podría estar subestimada y la masa de plata por partícula y los diámetros equivalentes sobrestimados.

Tabla 3.10. Resultados obtenidos por SP-ICP-MS para las distintas diluciones de la extracción alcalina para una muestra de heces. Media $\pm$ desviación estándar ($n=3$).

Muestra	Dilución	Tamaño equivalente más frecuente (nm)	LOD _{tamaño} (nm)	Concentración de Ag(I) ($\mu\text{g L}^{-1}$)	Nº de eventos obtenidos
Ag200	1:1000	46 ± 1	33.1	0.59 ± 0.01	4021 ± 24
	1:2500	37 ± 1	28.2	0.19 ± 0.01	2776 ± 63
	1:5000	32 ± 1	22.9	0.07 ± 0.01	1698 ± 8

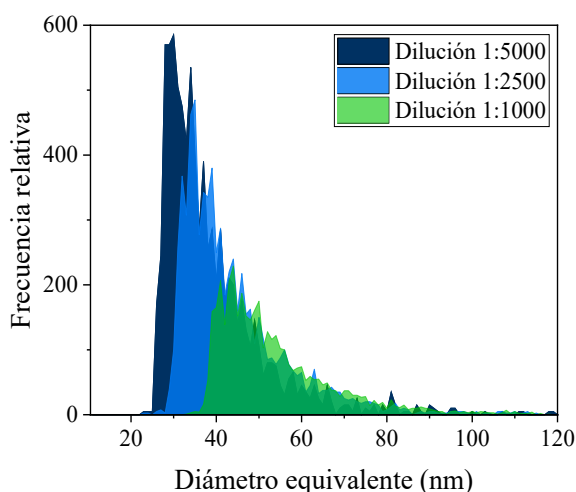


Figura 3.12. Comparativa de las distribuciones de tamaño obtenidas por SP-ICP-MS para las distintas diluciones de la extracción alcalina de una muestra de Ag200.

En la bibliografía, esta extracción alcalina se ha utilizado satisfactoriamente para extraer AgNPs en sangre y en tejido animal, ya que no modificaba su tamaño [16,21], sin embargo, se ha demostrado que puede provocar la precipitación de Ag(I) [22,23]. En nuestro caso, se comprobó que al adicionar Ag(I) sobre una muestra control de heces, aparecían partículas en las distribuciones (figura 3.13), lo que indicaría la formación de partículas a partir de Ag(I) durante la extracción alcalina. Casi un 4% de la plata total recuperada lo hizo en forma particulada. La formación de partículas podría deberse no

solo al TMAH, sino también a la presencia de la matriz, como ya describieron Vidmar et al. [22].

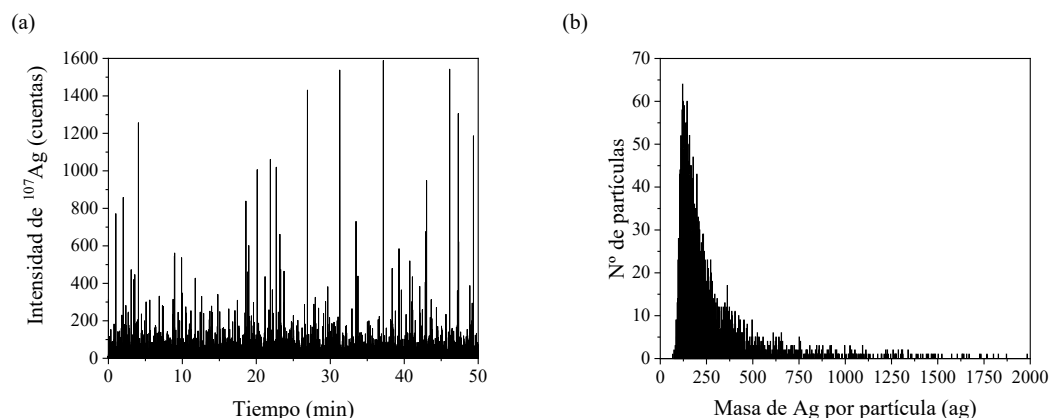


Figura 3.13. Resultados obtenidos por SP-ICP-MS para la extracción alcalina de la adición de Ag(I) sobre muestras control de heces. (a) Barrido de intensidad (b) Distribución en masa.

Se comprobó también que la extracción alcalina de una muestra control a la que se había adicionado AgNPs de 40 nm presentaba una distribución de tamaño (figura 3.14) cuyo diámetro más frecuente fue 40 ± 1 nm que era el de las nanopartículas adicionadas, confirmando que el TMAH extrae las nanopartículas intactas. Por lo tanto, se puede deducir que esta extracción sería apropiada para la extracción de AgNPs y no en el caso de Ag(I).

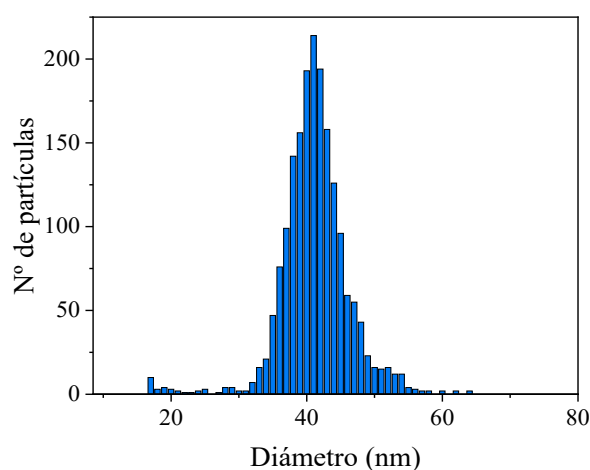


Figura 3.14. Distribución de tamaño para la extracción alcalina de la adición de AgNPs de 40 nm sobre una muestra control por SP-ICP-MS.

3.2.2.2. Determinación de las distintas especies de plata en la extracción alcalina por HDC-ICP-MS

El cromatograma obtenido para el sobrenadante de la extracción alcalina de una muestra de heces por HDC-ICP-MS se muestra en la figura 3.15. Se observó la aparición de un solo pico, que correspondería a Ag(I). No se observó la aparición del pico correspondiente a partículas de plata, puesto que, como se vio por SP-ICP-MS (tabla 3.9), la concentración de partículas era muy pequeña (en torno a 23.3 ng L⁻¹ para Ag20 y 92.7 ng L⁻¹ para Ag200), por debajo del LOQ de la técnica (17 µg L⁻¹).

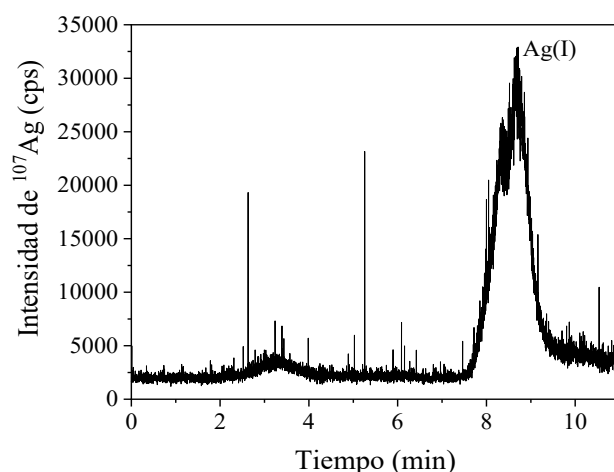


Figura 3.15. Cromatograma obtenido por HDC-ICP-MS para la extracción alcalina de una muestra de Ag20.

3.2.2.3. Determinación de las distintas especies de plata en la extracción alcalina por TEM

Los sobrenadantes resultantes de la extracción alcalina con TMAH, cisteína y Triton X-100 se analizaron por TEM sin ninguna dilución.

Debido a la película orgánica que recubría la muestra y al TMAH utilizado en la extracción, que es una base fuerte, capaz de atacar las placas de cobre sobre las que se depositaba la muestra, fue complicado ver con claridad las partículas. Sin embargo, fue posible ver algunas partículas que contenían plata. Al analizarlas por EDS, se observó una pequeña cantidad de S (figura 3.16), por lo que se puede deducir que las partículas de

plata pueden estar asociadas con S, probablemente como Ag_2S . El tamaño de las partículas encontradas se midió con el software Image-J, obteniéndose tamaños entre 20 y 40 nm (figura 3.17).

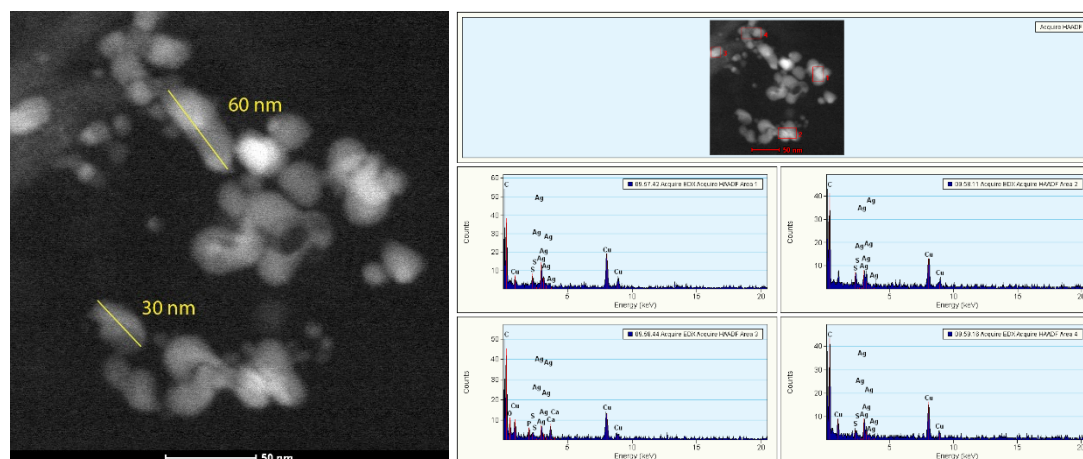


Figura 3.16. Imagen de una muestra de Ag_{200} obtenida por TEM y su correspondiente análisis por EDS.

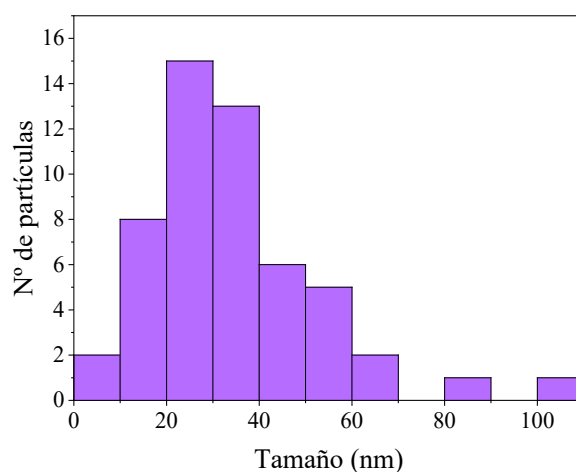


Figura 3.17. Distribución de tamaño de las partículas de plata obtenidas por TEM para la extracción alcalina.

3.2.3. Extracción secuencial con TSPP

Hong et al. [30] desarrollaron un método de extracción secuencial para fraccionar plata en suelos a los que se había adicionado plata disuelta (Ag(I)) y formas particuladas de plata metálica (AgNPs), cloruro de plata (AgCl-NP) y sulfuro de plata ($\text{Ag}_2\text{S-NP}$). En este método de extracción se emplearon TSPP, amoníaco y Na_2S para separar las distintas fracciones de plata. Se ha aplicado esta extracción secuencial a nuestras muestras de heces

obteniéndose un porcentaje total de plata extraída (sumando todas las fracciones obtenidas) de $23.2 \pm 7.4\%$ ($n=4$). Este porcentaje corresponde a una concentración de $31.7 \pm 7.9 \text{ mg kg}^{-1}$ ($n=2$) de plata total extraída para las muestras de Ag20 y $269 \pm 13 \text{ mg kg}^{-1}$ ($n=2$) para las muestras de Ag200. En la figura 3.18 se muestra la distribución de las fracciones de plata extraídas en las muestras analizadas. La mayor parte de la plata extraída lo hizo en forma de $\text{Ag}_2\text{S-NP}$ (45%), mientras que Ag(I) representó el 34% de la plata total extraída. El resto de la plata lo hizo como AgCl-NP (23%) y, solo un 6.6% lo hizo como AgNPs .

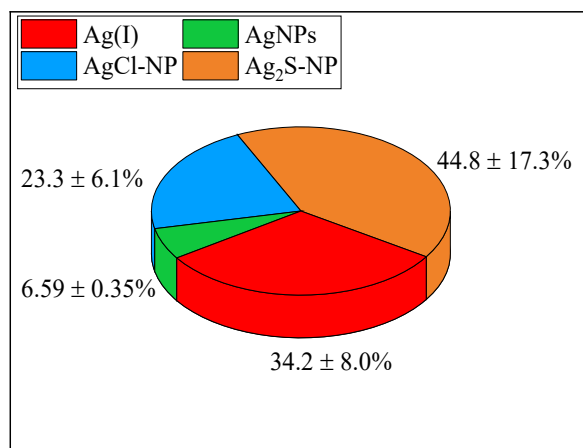


Figura 3.18. Porcentaje de las especies de plata liberadas durante la extracción secuencial con TSPP, amoníaco y Na_2S . Media $\pm$ desviación estándar ($n=4$).

El porcentaje total de extracción obtenido fue menor al obtenido en la extracción alcalina (42% aproximadamente) (sección 3.2.2). Esto se debe a que el TMAH contribuye a una mejor digestión de la matriz orgánica, al contrario que el TSPP [43]. La ventaja que tiene el procedimiento basado en extracción con TSPP frente a las utilizadas anteriormente radica en la posibilidad de fraccionar las diferentes especies de plata presentes en la muestra.

Hong et al. [30] consiguieron una eficiencia de extracción de entre 70 y 110% para muestras de suelos sobre las que se añadieron las distintas especies de plata, mientras que, con nuestras muestras, se extrajo menos del 30% de la plata total presente. Esto puede deberse a que este protocolo se desarrolló y optimizó para muestras de suelos, a las que

se les ha añadido plata, pero en nuestro caso, la plata ya estaba presente en las muestras. De hecho, cuando se aplicó este protocolo a muestras control de heces a las que se había adicionado Ag(I) y AgNPs, los valores de recuperación obtenidos estuvieron en torno al 77-88% (tabla 3.11), lo que coincide con los resultados que obtuvieron Hong et al. [30] en su artículo, donde recuperaron la totalidad de la plata adicionada a muestras de suelos, en cada una de las etapas. Además, la matriz de las heces es diferente a la de los suelos en cuanto a contenido en materia orgánica, pH, etc.

Tabla 3.11. Recuperaciones obtenidas por ICP-MS tras la extracción con TSPP, amoníaco y Na₂S para muestras control con adición de Ag(I) y AgNPs. Media $\pm$ desviación estándar ($n=3$).

Especie añadida	Recuperación (%)
Ag(I)	77.4 $\pm$ 1.4
AgNPs de 40 nm	88.3 $\pm$ 11.0

A la vista de estos resultados, se observa que la muestra real, en la que las uniones entre la plata y la matriz son más fuertes que si se adicionan los estándares sobre muestras control, se comporta de diferente manera, por lo que para valorar este método de extracción es necesario evaluar no sólo la recuperación de patrones añadidos, sino la recuperación de la plata total presente en las muestras originales. Bai et al. [31] optimizaron este método para la extracción de diferentes nanopartículas de plata en muestras de suelos, sedimentos y lodos, siendo capaces de extraer y diferenciar AgNPs, AgCl-NPs y Ag₂S-NPs, aunque no reflejan valores de eficiencia de extracción. Al realizar adiciones de plata sobre estas muestras también obtuvieron valores de recuperación de 90%, aproximadamente.

Schwertfeger et al. [43] estudiaron la idoneidad de utilizar TSPP como agente extractante para AgNPs, sin embargo, para la Ag(I) se ha encontrado que se liberaba una baja concentración, obteniendo mejores resultados con TMAH debido a su mayor capacidad de digestión de la materia orgánica. Por el contrario, Li et al. [28], en su comparación de diferentes extractantes (agua ultrapura, NaNO₃, KNO₃ y TSPP) para AgNPs en suelos y sedimentos, obtuvieron, finalmente, que el método con mejores rendimientos de extracción era el TSPP. Similares resultados obtuvieron Moreno-Martín et al [44] en la

extracción de AgNPs adicionadas sobre lodos de depuradora, encontrando valores de recuperación de hasta el 70%.

3.3. Comparación entre métodos de extracción

En la extracción con agua ultrapura, se obtuvo una baja eficiencia de extracción (en torno al 3%), además de observarse cambios en AgNPs y Ag(I) durante el proceso. Con ello, es imposible asegurar que las especies liberadas corresponden a las originales en la muestra. Por esta razón, la extracción con agua no es un método adecuado para la extracción de plata en heces. Sin embargo, sí que lo es para evaluar la movilización de plata y su especiación en medio ambiente cuando las heces entran en contacto con el agua, evaluando así su potencial riesgo medioambiental.

Comparando los métodos de extracción de TMAH y TSPP (figura 3.19), se puede concluir que la extracción alcalina con TMAH, combinada con el uso de SP-ICP-MS, es útil para detectar la presencia de partículas de plata en heces, ya que no se observaron ni transformaciones ni oxidación de las AgNPs, aunque la eficiencia de extracción de plata total obtenida fue baja (42%).

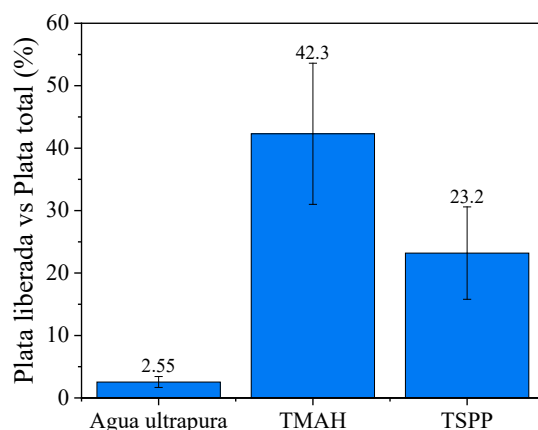


Figura 3.19. Comparativa de los resultados obtenidos por ICP-MS para los tres métodos de extracción para las muestras de heces ($n=3$).

Aunque los rendimientos de extracción obtenidos por el método de fraccionamiento con TSPP, amoníaco y Na_2S fueron inferiores a los de la extracción alcalina en muestras reales (figura 3.19), este método permitió la diferenciación de distintas fracciones de plata, como AgCl-NP y $\text{Ag}_2\text{S-NP}$, que no fue posible diferenciar con TMAH. Es importante subrayar que las especies de plata no se comportan de igual manera en las muestras reales que en el caso de las adiciones sobre muestras control, por lo que es necesario evaluar los métodos con muestras reales que contengan originalmente plata.

4. Conclusions

In vivo tests allow the direct assessment of the effect of a feed additive on the animals themselves and, in our case, to study the accumulation and elimination of silver in tissues, faeces and slurries of pigs fed with feed supplemented with Ag-kaolin. From the *in vivo* experiment, it was observed that silver was accumulated preferably in the liver and to a lesser extent, in the kidneys, not in the muscle, as well as that it was mostly eliminated through faeces and slurries.

To know in which forms the silver was in faeces and evaluate its release and environmental impact, three extraction methods have been studied. The extraction with water is the typical method for simulating the release of chemical substances into the environment. With this extraction, values of release less than 3% were obtained, predominant dissolved species of Ag(I) versus particulate forms. Changes in the silver species during the process were observed, which means that this method is not appropriate for the extraction of silver species in faeces, although it is useful for the study of the possible release of silver into the environment. The alkaline extraction with TMAH allows the digestion of the organic matter, as well as the extraction of the silver particles, which are released intact. The percentage of extraction of total silver with TMAH was 42%, approximately. The sequential extraction based on TSPP, NH_3 and Na_2S resulted in the fractionation of the different species of silver in a fraction of soluble Ag(I) and three particulate fractions: AgNPs , AgCl-NPs y $\text{Ag}_2\text{S-NPs}$, which is the difference from the rest of the used extractions.

Although this last extraction method has allowed the extraction of the different fractions of the silver species, with none of the methods used a high extraction efficiency was

obtained. These results reveal the difficulty of characterizing species derived from a nanomaterial in complex samples, such as faeces. The low extraction values obtained when analysing real samples imply the need to analyse this type of sample instead of the analysis of control samples on which silver species are spiked since the behaviour between them is different.

The use of different analytical techniques allowed to obtain a broader picture of the different species of silver released. Using HDC-ICP-MS and AF4-ICP-MS allowed the speciation of Ag(I) and silver particles (both high and low molecular weight). By SP-ICP-MS, the presence of smaller silver particles was also verified, although the high content of dissolved silver prevented the sight of smaller particles. By TEM-EDS the presence of these particles, in addition to the appearance of S, was confirmed.

4. Conclusiones

Los ensayos *in vivo* permiten evaluar directamente el efecto de un aditivo alimentario sobre los propios animales y, en nuestro caso, estudiar la acumulación y eliminación de plata en tejidos, heces y purines de cerdos alimentados con pienso suplementado con Ag-caolín. A partir del ensayo *in vivo* realizado se observó que la plata se acumulaba preferentemente en el hígado y en menor medida en los riñones, no haciéndolo en el músculo, así como que se eliminaba mayoritariamente a través de heces y purines.

Para conocer en qué formas se encontraba la plata en las heces y evaluar su liberación e impacto medioambiental, se han estudiado tres métodos de extracción. La extracción con agua es un método típico para simular la liberación de sustancias químicas al medio ambiente. Con esta extracción, se obtuvieron valores de liberación de plata total inferiores al 3%, predominando especies disueltas de Ag(I) frente a formas particuladas. Se observaron cambios en las especies de plata, lo que hace que este método no sea apropiado para la extracción cuantitativa de plata en heces, aunque sí es útil para el estudio de su liberación al medio ambiente. La extracción alcalina con TMAH permite la digestión de la materia orgánica, así como la extracción de partículas de plata, que se liberan intactas. El porcentaje de extracción de plata total con TMAH fue de 42%,

aproximadamente. La extracción secuencial basada en TSPP, NH_3 y Na_2S permite el fraccionamiento de las distintas especies de plata en una fracción de Ag(I) soluble y tres fracciones particuladas: AgNPs , AgCl-NPs y $\text{Ag}_2\text{S-NPs}$, lo que la diferencia del resto de extracciones utilizadas.

A pesar de que este último método de extracción ha permitido la extracción de diferentes fracciones de plata, con ninguno de los métodos utilizados se obtuvo una alta eficiencia de extracción. Estos resultados revelan la dificultad de caracterizar especies derivadas de un nanomaterial en muestras complejas, como las heces. Los bajos valores de extracción que se obtienen al analizar muestras reales implican la necesidad de analizar este tipo de muestras frente al análisis de muestras control sobre las que se adicionan especies de plata, ya que el comportamiento entre ellas es diferente.

La utilización de distintas técnicas analíticas permitió obtener una imagen más amplia de las distintas especies de plata liberadas. Mediante HDC-ICP-MS y AF4-ICP-MS permitieron la especiación de Ag(I) y partículas de plata (tanto de alto como de bajo peso molecular). Mediante SP-ICP-MS, también se comprobó la presencia de partículas de plata más pequeñas, aunque el alto contenido de plata disuelta impidió detectar las partículas más pequeñas. Por TEM-EDS se pudo confirmar la presencia de estas partículas con plata y su asociación con azufre.

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Chapter 4. Ecotoxicity of Ag-kaolin, feed and pig faeces to green algae

Abstract

In previous chapters, Ag-kaolin, a nanomaterial intended to be used as a growth-promoting additive in animal feed, has been studied following risk assessment strategies. In Chapter 3, it was confirmed that most of the silver from the nanomaterial was eliminated through the faeces. In such context, this chapter aimed to assess the ecotoxicity of the faeces, since they will lead to the corresponding slurries, which act as a silver source to the environment when used as fertilizers in agriculture. Additionally, the ecotoxicity of the nanomaterial and the supplemented feed was also studied. To this end, green algae *Raphidocellis subcapitata* (*R. subcapitata*) was exposed to leachates of Ag-kaolin, supplemented feed and pig faeces. A silver concentration of $10 \mu\text{g L}^{-1}$ in the leachate of Ag-kaolin led to the complete growth inhibition of the algae, producing a loss of chlorophyll fluorescence. Supplemented feed leachates required silver concentrations of $50 \mu\text{g L}^{-1}$ for complete growth inhibition, and no loss of chlorophyll fluorescence was found. Nevertheless, faeces leachates required silver concentration over $50 \mu\text{g L}^{-1}$ to reach total growth inhibition. When compared to pristine standards of Ag(I), $10 \mu\text{g L}^{-1}$ produced a loss in the chlorophyll fluorescence and led to complete growth inhibition. However, higher concentrations of AgNPs were required to obtain similar effects. Nonetheless, the silver ecotoxicity depended on the algae species, since *Chlamydomonas reinhardtii* (*C. reinhardtii*) was found to be less sensitive to silver than *R. subcapitata*, requiring higher concentrations to achieve similar inhibition effects.

1. Introduction

As was explained in the previous chapters, the silver-based nanomaterial Ag-kaolin was used as a growth-promoting additive in pig feed, due to the antibacterial properties of the silver [1–3]. After administration of the supplemented feed to animals, silver could reach the environment through their faeces. As it was presented in the previous chapter when faeces come into contact with water, a low amount of silver (3.75 and 20.3 mg kg^{-1} for Ag20 and Ag200 faeces, respectively) was released, mostly as Ag(I). Additionally, silver

bound to particles with sizes larger than 300 nm was observed by FESEM-EDS. Moreover, the presence of sulphur was confirmed, which means that silver could be present as Ag₂S [4]. Ultimately, these materials could be found in the aquatic environment, although their potentially harmful effects have yet to be determined.

Photoautotrophic microalgae are one of the basic single-celled organisms and the primary producers in aquatic systems [5]. They are the first organisms vulnerable to aquatic contaminants and, therefore, would affect the heterotrophs, since they are the first link of the accumulation of heavy metals in the food chain in aquatic systems [6,7]. The toxicity for microalgae of several heavy metals [8] and nanoparticles [9,10] has been studied. One of the most studied metals is silver (both as Ag(I) and AgNPs), due to the increase in its use, which leads to a higher presence in the environment [8]. Several articles have studied its toxicity in several green algae, such as *R. subcapitata* [11–17], *C. reinhardtii* [10,17–21] or *Daphnia magna* (*D. magna*) [12,22,23], among others. Ribeiro et al. [12] studied the toxicity of Ag(I) and AgNPs for *R. subcapitata*, finding that Ag(I) was more toxic to algae than AgNPs. Indeed, they found that 50 µg L⁻¹ of Ag(I) inhibited the growth of the algae by 100%, whereas 50 µg L⁻¹ of AgNPs inhibited the growth by 75%. Similar results were found by Sakamoto et al. [22] for *D. magna*, finding a half maximal effective concentration (EC₅₀) higher for AgNPs (0.90 µg L⁻¹) than for AgNO₃ (0.25 µg L⁻¹); and by Khoshnamvand et al. [23] for *Chlorella vulgaris*, finding values for EC₅₀ of 20.1 ± 1.5 µg L⁻¹ for the Ag(I) against 33.6 ± 2.1 µg L⁻¹ obtained for AgNPs.

One reason for this toxicity is the internalization of silver by algae cells. A review published by Mahana et al. [24] summarized different methods for nanoparticle internalization by algae: interacting with the cell membrane, endocytosis, protein-coupled transport across the membrane or using membrane-bound proteins as carriers. It was proposed that Ag(I) is uptaken by cells through the same channel as Cu(I) [21] because cells could not distinguish between these two ions [11]. In addition, inside the cells, the presence of silver leads to the formation of ROS, harming photosynthetic yield. The harm to the photosynthetic yield is related to the displacement of trace metals by Ag(I), which binds to thiol groups of the proteins, making them non-functional. Liu et al. [25] found that AgNPs decreased the enzymatic activity of human catalase by 60% after 24 h of exposition due to the bound to these functional groups. The partial inhibition of ferroxidase activity was also reported [26]. The bound to proteins also could lead to the

displacement of Cu(I), which caused the inactivation of the photosynthetic electron transport, as was observed in *C. reinhardtii* [8,27,28]. In addition, the presence of ROS causes oxidative stress, which results in damage to the membranes by the peroxidation of lipids, observed by the increase in the concentration of malondialdehyde. Even at low concentrations, a defence response against ROS was observed [27,28]. In addition, the toxicity of silver leads to changes in the morphology of the cells [29,30] and the loss of cell membrane integrity [27,28,31]. The changes in cell morphology can be explained again by the interactions between silver and the thiol groups of glycoproteins of the membrane cell, inhibiting enzymatic activity [8]. Some authors have reported that cells do not internalize AgNPs, but all the silver which is found inside is Ag(I) directly incorporated from the oxidative dissolution of AgNPs [11,20]. In fact, Leclerc et al. [21] found the same silver deposits for *C. reinhardtii* when exposed to Ag(I) or AgNPs, which suggested that the cells did not internalize NPs and Ag(I) precipitated inside the cells. On the contrary, Liu et al. [28] found high aggregates of AgNPs inside vacuoles when *Poteroiochromonas malhamensis* (*P. malhamensis*) were exposed to AgNPs, suggesting that AgNPs were uptaken by phagocytosis. However, they pointed out that it was not clear if the mechanism of AgNPs toxicity was caused directly by them or indirectly by the release of Ag(I). Also, AgNPs which were not internalized could form silver aggregates with different sizes externally, which was another effect of silver exposition. This could induce physical changes and interfere with their growth or lead to faster sedimentation of the cells [11].

The toxicity of AgNPs depends on the size, shape and coating of the particles [13–15,20,32]. Smaller particles have more toxicity than larger ones [14]. The dependence of the coating is related to the possibility of association of the nanoparticles with the organic matter, which can affect their aggregation, dissolution and toxicity [13]. Worms et al. [33] showed that humic substances and the concentration of free metal ions, affected the uptake of different metals by *C. reinhardtii*. The exposure time also influences the toxicity of the silver [15,17,28]. Hiriart-Baer et al. [17] observed that large exposure times (18-36 h) could benefit an acclimation response by algae, leading to a decrease in the uptake rate, which, in turn, depends on the concentration of silver as well. Likewise, the culture medium of the algae affects the toxicity effect. Actually, the presence of other chemical groups, such as sulphide or chloride, can modify the toxicity of silver [12,13,15,17].

Silver, in presence of thiosulphate, was less toxic because silver thiosulphate complexes inside the algae were intact in the intracellular environment, causing a low bioavailability of silver [15,17]. The formation of silver chloride decreased the toxicity of silver, [9,12], due to the formation of the precipitates, as in the case of sulphide, which led to lower bioavailability of Ag(I) [12].

Despite the advances in the knowledge of the toxicity of AgNPs on the microalgae, the current knowledge is obtained with standard AgNPs, and information is lacking about the possible effect of complex AgNPs-containing materials when released in the aquatic environment. The aim of the work in this chapter has been the study of the ecotoxicity of the faeces from pigs fed with feed supplemented with Ag-kaolin in a previous *in vivo* study. Since pig slurries, typically used as fertilisers in agriculture, were not available, faeces were used instead. The toxic effects of the original NM, Ag-kaolin, and the supplemented feed were also studied. For this objective, the effects of these materials on green algae *R. subcapitata*, including the effect on algal growth, chlorophyll autofluorescence and cell morphology, were also studied. Flow cytometry and fluorescence microplate reader were used, as well as SC-ICP-MS to study the absorption of silver by the cells. Additionally, a comparison of the effect of the samples in two green algae (*R. subcapitata* and *C. reinhardtii*) was also carried out.

2. Materials and methods

2.1. Instrumentation

The impact of different materials on algal cell growth and fluorescence was assessed using a BD Accuri C6 Plus flow cytometer (BD, New Jersey, USA). The flow rate used was 35 $\mu\text{L min}^{-1}$ and the core size was 16 μm .

A Multifuge X4 Pro Centrifuge Series (Thermo Fisher, New Jersey, USA) was used for the preconcentration of the algae and the separation of the solid and liquid in the leaching process.

For the measurement of the chlorophyll fluorescence and to take images of the algae, a Cytation 3 Image Reader Biotek (Santa Clara, USA) microplate reader and the software Gen 5 version 3.11 were used. For the obtention of the images with more resolution, the

optical microscope Olympus BX61 (Olympus, Tokyo, Japan), with CellSens Dimension software, were also used. For the analysis of the fluorescence, a pE-300 module from CoolLED (CoolLED Ltd, New York, USA) was utilized.

For the analysis by SC-ICP-MS, a Perkin Elmer NexION 2000 (Perkin Elmer, Toronto, Canada) was used. The sample introduction was an AsperonTM linear pass spray chamber (Perkin Elmer, Toronto, Canada) with a flow-focusing nebulizer (Ingeniatrics, Sevilla, Spain). The instrument was optimized daily with a solution with a concentration of 1 $\mu\text{g L}^{-1}$ of Be, Ce, Fe, In, Li, Mg, Pb and U, provided by the manufacturer. In addition, the acquisition parameters for the silver were also optimized to achieve the maximum sensitivity for this element, with a solution of 1 $\mu\text{g L}^{-1}$ of Ag(I) in 1% of HNO_3 , prepared daily. The conditions used are shown in table 4.1.

Table 4.1. Acquisition conditions for the analysis by SC-ICP-MS.

ICP-MS parameters	
RF Potency (W)	1600
Argon gas flow	
Plasma (L min^{-1})	15
Auxiliar (L min^{-1})	1.2
Nebulizer (L min^{-1})	1.0
Make-up (L min^{-1})	0.2
Sample flow rate ($\mu\text{L min}^{-1}$)	13
SC-ICP-MS	
Dwell time (μs)	100
Readings per replica	500000
Settling time (ms)	0
Acquisition time (s)	50
Monitored isotopes	^{107}Ag
Transport efficiency	10.0

2.2. Chemicals

The algae were incubated in a typical Tris-acetate-phosphate (TAP) medium with Hutner's trace elements solution [34]. For its preparation, ammonium chloride (NH_4Cl), calcium chloride dihydrated ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), potassium phosphate dibasic (KH_2PO_4) potassium phosphate monobasic (K_2HPO_4), Trizma base ($\text{C}_4\text{H}_{11}\text{NO}_3$) (Sigma-Chemie, Sta. Louis, USA), magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) (Merck, Darmstadt, Germany) and acetic acid (Acros organics, Thermo Fisher, New Jersey, USA) were used. For the Hutner's metal trace solution, EDTA- Na_2 , boric acid (H_3BO_3), zinc sulphate

heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), manganese chloride tetrahydrate ($\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$), iron sulphate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), cobalt chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$), copper sulphate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) and ammonium molybdate tetrahydrate ($(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$) (Sigma-Chemie, Sta. Louis, USA) were needed.

For the preparation of the TAP exposure medium, KH_2PO_4 and K_2HPO_4 , ammonium nitrate (NH_4NO_3) and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Sigma-Chemie, Sta. Louis, USA), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Merck, Darmstadt, Germany) and acetic acid (Acros organics, Thermo Fisher, New Jersey, USA) were used. To reach the pH of 7 a solution of NaOH (Sigma-Chemie, Sta. Louis, USA), was used.

A standard solution of Ag(I) with a concentration of 1000 mg L^{-1} (Sigma-Chemie, Sta. Louis, USA) was utilized. Suspension of AgNPs with a size of $50 \pm 4 \text{ nm}$ (Nanocomposix, San Diego, CA, USA) and a concentration of 1.07 mg mL^{-1} (1.5×10^{12} particles mL^{-1}) was also used.

For the determination of the transport efficiency for SC-ICP-MS, a microparticle size standard based on polystyrene (PS) monodisperse with a size of $10 \pm 0.15 \mu\text{m}$ and 2% of solid content (Fluka, Steinheim, Germany) was used.

2.3. Samples

Three different samples were used: Ag-kaolin, supplemented feed and faeces (both Ag20 and Ag200). Faeces Ag20 correspond to pigs fed with feed which contained 20 mg kg^{-1} of silver and faeces Ag200 correspond to pigs fed with 200 mg kg^{-1} . The total content of silver in the samples and the total silver released in water, obtained in the previous chapters, are in table 4.2.

Table 4.2. Total silver content and total silver released in water for the samples.

Sample		Total silver content	Total silver released in water
Ag-kaolin		$8.32 \pm 0.35 \text{ mg g}^{-1}$	$2.94 \pm 0.06 \text{ mg g}^{-1}$
Feed		$12.6 \pm 1.1 \text{ mg kg}^{-1}$	$0.721 \pm 0.107 \text{ mg kg}^{-1}$
Faeces	Ag20	$155 \pm 30 \text{ mg kg}^{-1}$	$3.75 \pm 1.13 \text{ mg kg}^{-1}$
	Ag200	$1026 \pm 178 \text{ mg kg}^{-1}$	$20.3 \pm 4.3 \text{ mg kg}^{-1}$

2.4. Algae

The algae used in this work were *R. subcapitata* and *C. reinhardtii* due to their high sensitivity to silver.

R. subcapitata is a green alga with a characteristic “C” shape (figure 4.1) with a size between 5 and 12 μm . If they are healthy, they have autofluorescence emission at 670 nm due to chlorophyll.

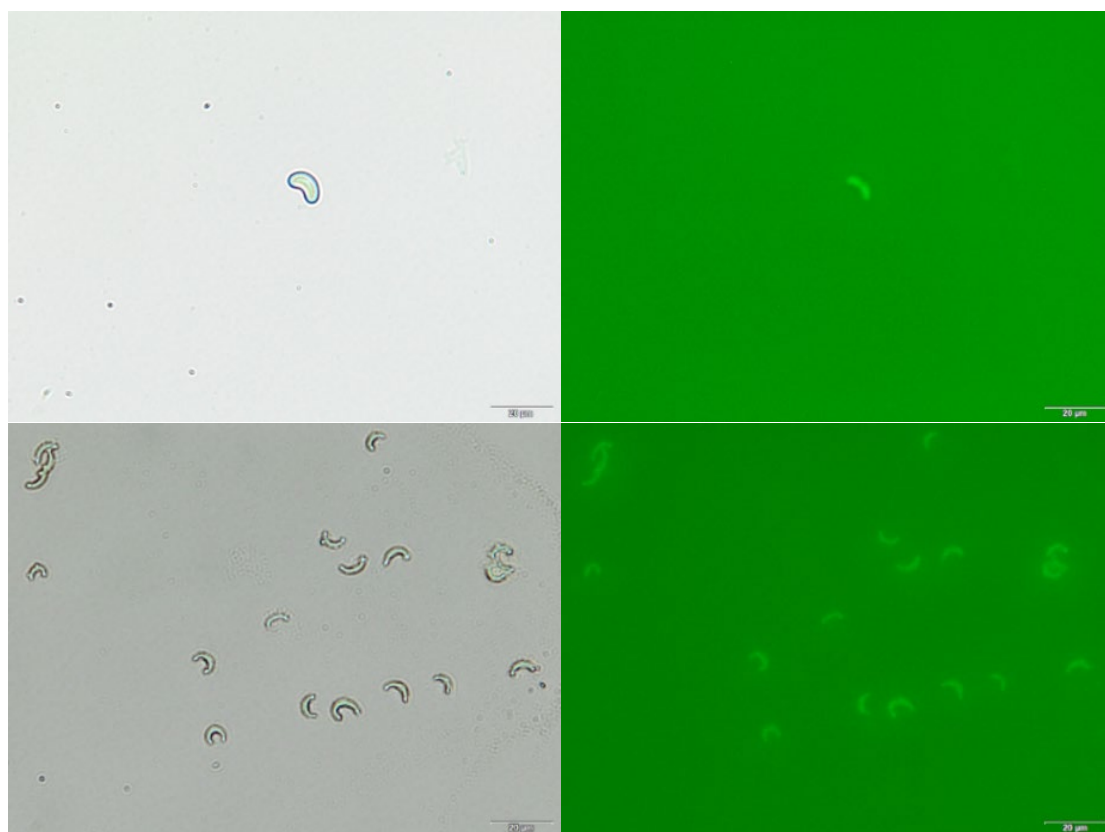


Figure 4.1. Images of *R. subcapitata* and the chlorophyll fluorescence measured by optic microscope.

C. reinhardtii is a unicellular alga with a spherical shape with two short cilia at one pole, and a diameter between 5 and 10 μm (figure 4.2) [35]. If they are healthy, they have also autofluorescence emission at 670 nm due to chlorophyll.

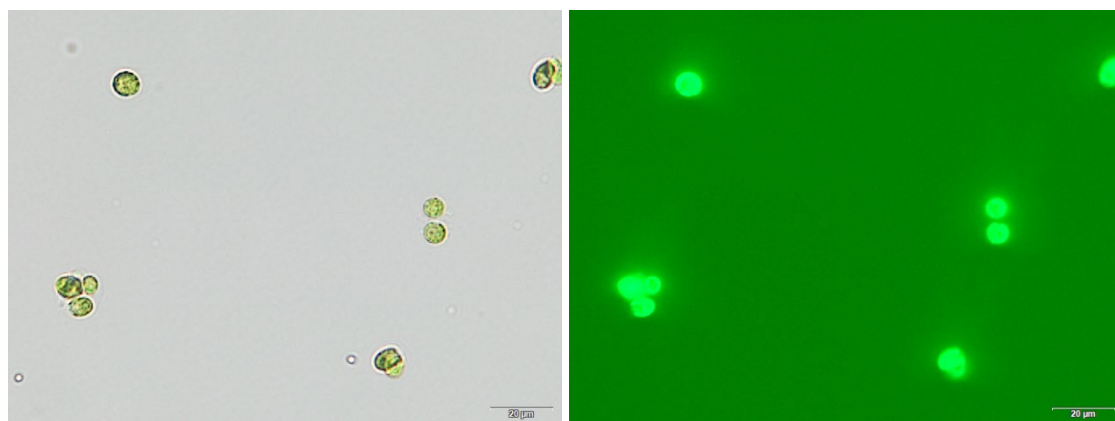


Figure 4.2. Images of *C. reinhardtii* and the chlorophyll fluorescence measured by optic microscope (size: 6-8 μm).

2.5. Procedures

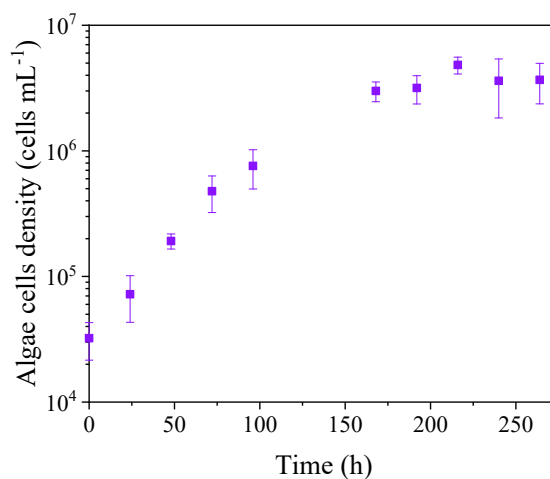
All the materials used were previously autoclaved with an autoclave Zirbus LVSA 50-70 (ZIRBUS technology GmbH, Bad Grund, Germany), at 120 °C for 30 minutes.

2.5.1. Algae cultivation

The algae were cultivated in TAP medium with the Trace Elements solution, whose composition is shown in table 4.3, for 11 days in an incubator (Binder GmbH, Tuttlingen, Germany) (temperature: $20.1 \pm 0.1^\circ\text{C}$, light programme: 16 hours of light and 8 hours of dark) to obtain the growth curve, which is shown in figure 4.3. The culture was renewed every 11 days, resuspending them with a new medium. To obtain the growth curve, the density of cells was measured by flow cytometry every 24h. Between the inoculation day and the 6th day, the proliferative phase is occurring. Between the 6th day and the 11th day, the stationary phase is happening. In this phase, the cells start to age and after that, they enter the death phase, where they lose the integrity of the membrane [36].

Table 4.3. Composition of TAP medium.

Solutions	Concentration (g L ⁻¹)
5 x Beijerincks' Solution	
NH ₄ Cl	40
CaCl ₂ ·2H ₂ O	5
MgSO ₄ ·7H ₂ O	10
Phosphate Solution	
K ₂ HPO ₄	14.3
KH ₂ PO ₄	7.26
Tris-Acetate Stock Solution	
Tris base	242
Acetic Acid	0.1 % (v/v)
Trace Elements Solution	
EDTA-Na ₂	50
H ₃ BO ₃	11.14
ZnSO ₄ ·7H ₂ O	22.0
MnCl ₂ ·4H ₂ O	5.1
FeSO ₄ ·7H ₂ O	5.0
CoCl ₂ ·6H ₂ O	1.6
CuSO ₄ ·5H ₂ O	1.6
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	1.1
pH 7	

**Figure 4.3.** Growth curve of *R. subcapitata* in TAP medium.

The average growth rate (μ) was calculated following equation (4.1) [37], where μ_{i-j} is the average growth rate from time i to time j , X_i is the cell density at time i and X_j is the cell density at time j . The growth rate obtained after 11 days was $0.402 \pm 0.178 \text{ day}^{-1}$, similar to the obtained in the bibliography ($0.47 \pm 0.4 \text{ day}^{-1}$) [38].

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i} \text{ (day}^{-1}\text{)} \quad (4.1)$$

2.5.2. Algae growth inhibition

The ecotoxicity tests of the leachate of the different samples were carried out by the study of algae growth inhibition. The leachate of Ag-kaolin, feed and faeces in ultrapure water was performed for 1 h for Ag-kaolin and feed, and 48 h for faeces (as it was explained in the protocols in Chapter 2 (page 125) and Chapter 3 (page 170)). The supernatant was separated from the solid residue by centrifugation (7000 g for 17 min). The appropriate amount of the leachate, to expose the algae to an equivalent concentration of silver (0.1, 1, 10 and 50 $\mu\text{g L}^{-1}$), was added to the cultures.

The algae were cultivated for 72 h in TAP medium with an initial cell density of 5×10^4 cells mL^{-1} . After that, algae were preconcentrated and centrifuged (13000 g for 10 min) and the pellet was taken. The pellet was resuspended with 5 mL of TAP exposure medium, whose composition is shown in table 4.4, and the density of cells was quantified by flow cytometry. The initial cell density at the beginning of the exposition experiment was 3×10^5 cells mL^{-1} .

Table 4.4. Composition of TAP exposure medium.

Solutions	Concentration (g L^{-1})
Ammonium Solution	
NH_4NO_3	0.134
$\text{CaCl}_2 \cdot 4\text{H}_2\text{O}$	0.0150
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.0209
Phosphate Solution	
K_2HPO_4	0.0259
KH_2PO_4	0.0137
Acetate Stock Solution	
Glacial Acetic Acid	0.0245 % (v/v)
pH 7	

Then, a volume of the leachate of the samples, which depends on the concentration studied, was added to the cells to study the effect on their growth. The algae were left to grow for 72 h, controlling every 24 h the cell density and the autofluorescence. The

cultures were also analysed by fluorescence microscope to visualize the cell morphology and the loss of fluorescence.

To obtain the percentage of growth inhibition, equation (4.2) [37] was applied:

$$\%I_r = \frac{\mu_c - \mu_t}{\mu_c} \times 100 \quad (4.2)$$

where $\%I_r$ is the percentage of growth inhibition, μ_c is the average growth rate of the control and μ_t is the average growth rate of the treated cells.

2.5.3. Determination of cell density and fluorescence by flow cytometry

The cell density and the distribution of the chlorophyll autofluorescence were determined by flow cytometry. Flow cytometry is a technique based on the analysis of multiple parameters of single cells or particles, such as size or fluorescence. It uses lasers as light sources to generate scattering or fluorescence, which would be read by different detectors, like photodiodes or photomultiplier tubes. The detectors convert the light signal into an electrical current, which is finally converted into digital data, which can be displayed as histograms or plots [39,40].

To determine the cell density and ensure a minimum number of algae events in the algal window, to be able to discriminate between blank samples and algae suspensions, a minimum of 3×10^5 cells mL^{-1} was needed. To achieve that, no dilution was applied to the samples analysed. 200 μL of the samples were put in a tube and, before the analysis, they were shaken. 30 μL were introduced in the flow cytometer and the total events were collected. Before and after the analysis, ultrapure water was introduced into the instrument to clean it.

2.5.4. Determination of the chlorophyll fluorescence and visualization of the algae

50 μL of the suspensions of algae exposed to the leachate of the samples were put into 96 black/clear bottom microplates to read the chlorophyll fluorescence and visualize the cell morphology by the microplate reader. The plates were left for 30 minutes for the algae to settle into the bottom of the well and be able to visualize them clearly. The images were

taken with a 40x objective. The filter for the chlorophyll autofluorescence was also applied to see the fluorescence in the images.

2.5.5. Analysis of the algae exposed to silver materials by SC-ICP-MS

To study the uptake of silver by the cells, the suspensions of sample leachates were analysed by SC-ICP-MS. After 72 h of exposition experiments, the algae were lyophilized to store them until the analysis by SC-ICP-MS. Then, the algae lyophilized was suspended in 50 mL of ultrapure water and introduced directly to the instrument.

Before the analysis of algae, the sample flow rate ($13 \mu\text{L min}^{-1}$) and the transport efficiency were obtained. The transport efficiency was calculated following the frequency method, following the procedure described by Pace et al. [41]. To do that a suspension of $10 \mu\text{m}$ PS microparticles, with a concentration of 3.5×10^7 particles mL^{-1} , was used. A calibration curve of Ag(I) in 1% of HNO_3 , with standards of concentration between 0 to $1 \mu\text{g L}^{-1}$, was also measured.

Origin 9.6.5.169 (OriginLab Corporation, MA, Northampton, U.S.A.) software was used for the representation of the data.

2.5.6. Comparison of the ecotoxicity for two algae

To compare the ecotoxicity to silver between two green algae, *R. subcapitata* and *C. reinhardtii* were cultivated in a 96-well microplate, and they were exposed to Ag(I) and AgNPs standards solutions and to the leachates of Ag-kaolin, feed and faeces. The algae were exposed to different concentrations of silver: 0.1, 1, 10 and $50 \mu\text{g L}^{-1}$ for 72 h. Every 24 h, the chlorophyll fluorescence of the algae was read by the microplate reader, and after 72 h of exposition, the algae were visualised to see changes in the morphology and the chlorophyll fluorescence.

2.6. Data analysis

All the experiments were carried out in triplicate. One-way analysis of variance (ANOVA) was performed to test for significant differences between the different

treatments. A p-value < 0.05 was considered statistically significant. The Tukey Honestly Significant Difference was performed as a post-hoc test.

3. Results and discussion

3.1. Effect of different silver-containing materials on the algal growth

R. subcapitata was exposed to the leachates of the different samples described in section 2.3. Although the effect of Ag(I) and AgNPs has been widely studied in the bibliography, *R. subcapitata* was also exposed to standard solutions of Ag(I) and 50 nm AgNPs under the same conditions to compare the results obtained.

3.1.1. Ecotoxicity of Ag(I) and AgNPs standards

The growth rate and the inhibition obtained for the exposition to Ag(I) are shown in table 4.5. The toxicity of Ag(I) increased as the concentration of Ag(I) did, as it was demonstrated in the bibliography [15]. There was a low inhibition of the growth with a concentration of 0.1 and 1 $\mu\text{g L}^{-1}$ of Ag(I), but the inhibition was complete at 10 $\mu\text{g L}^{-1}$. Changes in the chlorophyll autofluorescence were also observed. The autofluorescence cytograms obtained for every concentration studied are shown in figure 4.4. In the cytograms, the distribution of the logarithm of the intensity of the chlorophyll fluorescence, with arbitrary units, is represented. For the concentrations of 0.1 and 1 $\mu\text{g L}^{-1}$, no loss of the intensity of the fluorescence was found. However, for the 0.1 $\mu\text{g L}^{-1}$, two populations appeared, as it is shown in the cytogram in figure 4.5. This cytogram represents the light scattered, by the logarithm of the side scatter (SSC), against the logarithm of the forward scatter (FSC). These parameters can be related to the size of the cells. The intensity of FSC is proportional to the size or the surface area of the cell and SSC is proportional to the internal complexity or the cell granularity [39]. Since the growth rate inhibition was very low for this concentration (less than 1%), this situation could be due to a stress situation that does not lead to cell death. For the concentration of 10 $\mu\text{g L}^{-1}$, the loss of the intensity of the fluorescence after 48 h of exposure was shown, and for the concentration of 50 $\mu\text{g L}^{-1}$, the loss of the intensity appeared already in the first 24 h and was higher as the time of exposition increased. As explained before, Ag(I) decreases the chlorophyll content due to the inhibition of its synthesis [8,27], which causes the death of the algae.

Table 4.5. Growth rate and percentage of growth rate inhibition for *R. subcapitata* exposure to standard solutions of Ag(I). Mean $\pm$ standard deviation ($n=3$)

Ag(I) added ($\mu\text{g L}^{-1}$)	Growth rate (day^{-1})	Growth rate inhibition (%)
0.1	0.421 ± 0.006	0.974 ± 0.001
1.0	0.387 ± 0.008	18.1 ± 1.8
10	0	100
50	0	100

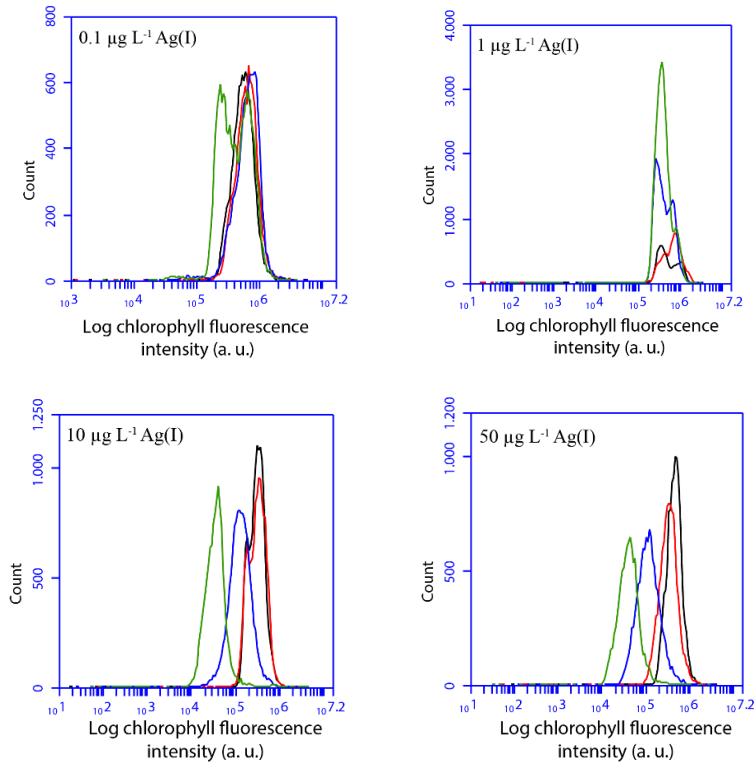


Figure 4.4. Autofluorescence cytograms for *R. subcapitata* during 72 h exposed to Ag(I). Black: 0 h. Red: 24 h. Blue: 48 h. Green: 72 h.

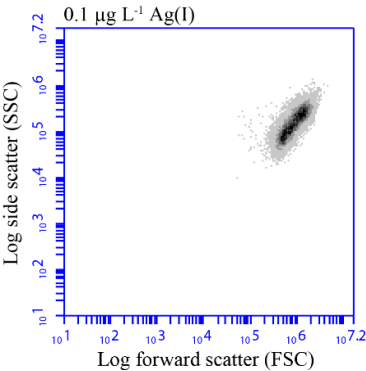


Figure 4.5. SSC/FSC dot-plot cytograms of *R. subcapitata* exposed $0.1 \mu\text{g L}^{-1}$ of Ag(I) for 72 h.

The suspensions were also measured by fluorescence microscopy to see the changes in the chlorophyll fluorescence and morphology. The images obtained after 72 h are shown in figure 4.6 (the images obtained every 24 h are in Appendix 3, figure A3.1). The cell density and the chlorophyll fluorescence (which is represented by the intensity of the red colour) decreased as the concentration of Ag(I) increased. With an exposition to a concentration of $10 \mu\text{g L}^{-1}$ of Ag(I), it was observed that the fluorescence was very low, compared to the concentration of $1 \mu\text{g L}^{-1}$. In the image of the exposition of the solution of $50 \mu\text{g L}^{-1}$ of Ag(I), a very low cell density was observed, and no fluorescence was found. Taylor et al. [31] found a decrease in the number of intact cells, with 60% of viable cells, after the exposition to Ag(I), due to damage in the cell membrane.

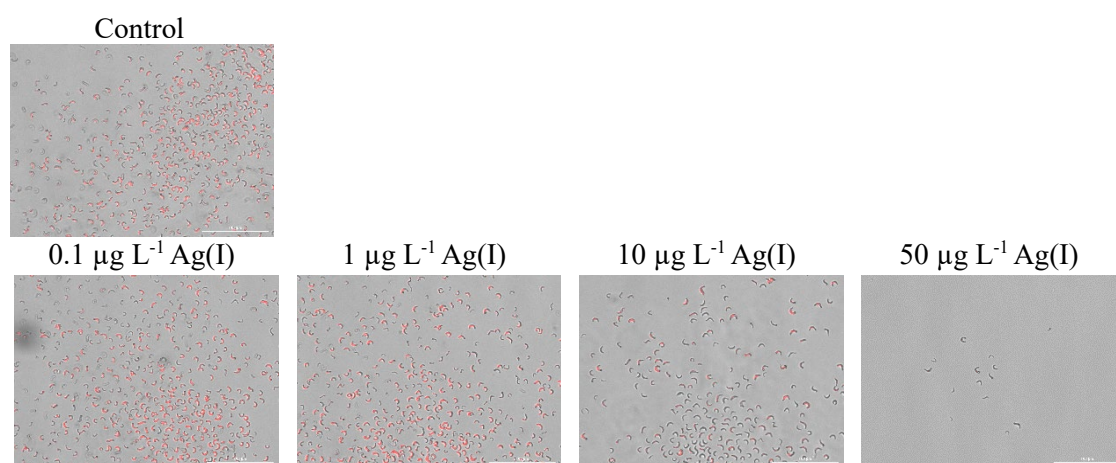


Figure 4.6. Chlorophyll fluorescence images for *R. subcapitata* after 72 h for algae exposed to standard solutions of Ag(I).

In the bibliography, similar results were found for *R. subcapitata*. Hiriart et al. [17] studied the effect of Ag(I) on *R. subcapitata* and found that the effects in the growth appeared at low concentrations and the growth was inhibited when the concentration of free silver exceeded 10 nM. Ribeiro et al. [12] found that the EC_{50} value for AgNO_3 was $33.79 \mu\text{g L}^{-1}$ and $50 \mu\text{g L}^{-1}$ of ionic silver caused growth inhibition of almost 100% for *R. subcapitata*.

R. subcapitata was also exposed to standard solutions of 50 nm AgNPs of different concentrations. The results obtained for the growth rate and the inhibition are shown in table 4.6. Unlike the results obtained for $50 \mu\text{g L}^{-1}$ Ag(I), the inhibition of the growth rate was 4%, and at least a concentration of $100 \mu\text{g L}^{-1}$ of AgNPs was needed to get an

inhibition of 90%. Ribeiro et al. [12] found that a concentration of $100 \mu\text{g L}^{-1}$ of AgNPs with alkane coating was needed to achieve 100% growth inhibition, in fact, at that concentration they found that AgNPs were lethal for *R. subcapitata*. Nevertheless, the toxic concentration depends on the organism studied. In the bibliography different concentration needed to inhibit the growth was found for other microorganisms, such as *Ochromonas danica* (*O. danica*) ($29 \mu\text{g L}^{-1}$ of carboxy-functionalized silver-engineered nanoparticles (Ag-EN) and $100 \mu\text{g L}^{-1}$ of AgNPs coated with PVP) [42,43], *C. reinhardtii* ($300 \mu\text{g L}^{-1}$) [42] or *C. vulgaris* ($\text{EC}_{50} 33.63 \pm 2.10 \mu\text{g L}^{-1}$) [23].

Table 4.6. Growth rate and percentage of growth rate inhibition for *R. subcapitata* exposed to 50 nm AgNPs. Mean $\pm$ standard deviation ($n=3$).

AgNPs added ($\mu\text{g L}^{-1}$)	Growth rate (day^{-1})	Growth rate inhibition (%)
0.1	0.623 ± 0.004	0
1.0	0.627 ± 0.006	0
10	0.607 ± 0.011	0.112 ± 0.002
50	0.585 ± 0.006	3.93 ± 1.10
100	0.0526 ± 0.0005	91.3 ± 0.9

In contrast to the exposure results in Ag(I) (figure 4.4), for the exposition to 50 nm AgNPs, with the same concentration, there was no loss of fluorescence (figure 4.7). Nevertheless, for a concentration of $100 \mu\text{g L}^{-1}$ of AgNPs, a loss of fluorescence after 48 h of exposition was shown, resulting in the formation of two populations of algae, one with the same fluorescence and the other with less intensity. After 72 h, there were still two populations, but the loss of fluorescence was higher. Zhao et al. [44] also found that the changes in the fluorescence were more evident after 48 h and 72 h of exposition of *C. reinhardtii* to AgNPs. The increase in the loss of fluorescence by AgNPs through time was also visualized by Nguyen et al. [45], seeing a decrease in the fluorescence from 98% to 22%, in 1 h and 24 h, respectively.

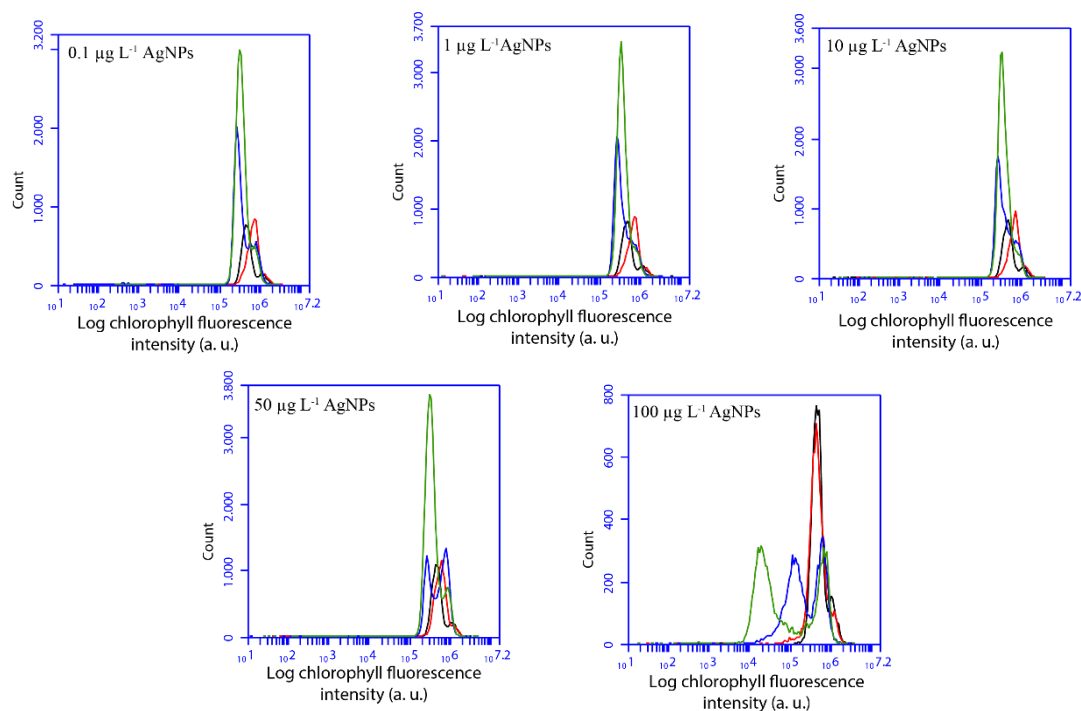


Figure 4.7. Autofluorescence cytograms for algae cells during 72 h exposed to 50 nm AgNPs. Black: 0 h. Red: 24 h. Blue: 48 h. Green: 72 h.

In figure 4.8, the images obtained by the microscope after 72 h are shown. They show a loss of chlorophyll fluorescence when the algae were exposed to a concentration of 100 $\mu\text{g L}^{-1}$, and a visible decrease of the cell density at that concentration, which agrees with the results obtained by flow cytometry (table 4.6). Zhao et al. [44] obtained that the total content of chlorophyll of *C. reinhardtii*, decreased after the exposition of AgNPs, increasing the toxicity as the concentration of AgNPs increased. The decrease in the content of chlorophyll indicated that photosynthesis was inhibited, which is an ecotoxicity effect of AgNPs in aquatic systems. Qiao et al. [46] found that the inhibition of chlorophyll synthesis is higher as the concentration increases. Ag(I) released from the AgNPs also affects the photosynthetic yield [28,44], inhibiting the synthesis of chlorophyll.

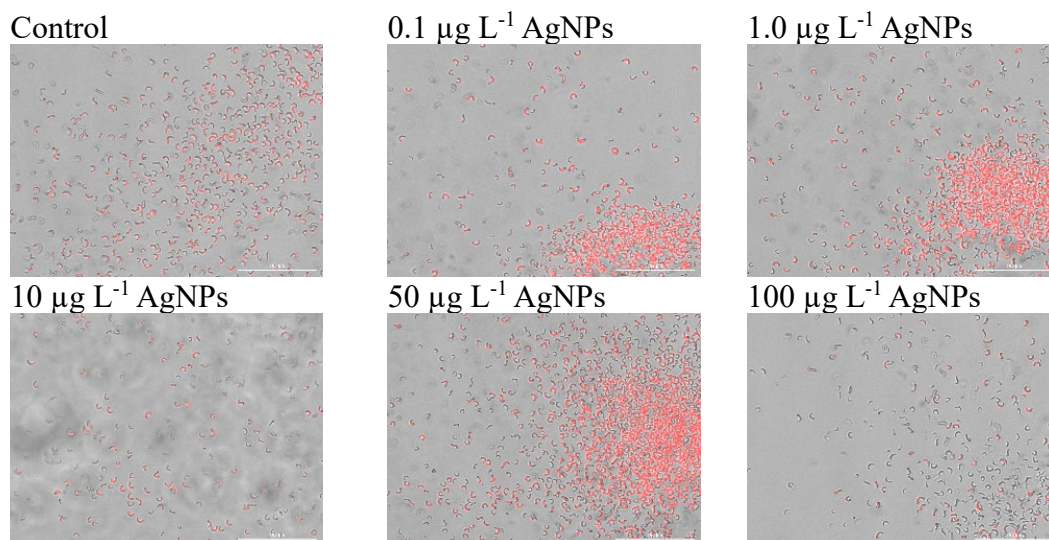


Figure 4.8. Chlorophyll fluorescence images for *R. subcapitata* after 72 h exposed to standard solutions of 50 nm AgNPs.

After the application of ANOVA, it was concluded that the behaviour of Ag(I) and AgNPs showed significant differences for all the concentrations. These results are consistent with the existing literature, in which AgNPs were found less toxic than Ag(I) [12,14,15,23,43]. The toxicity of the AgNPs could be related to their uptake [43] and Ag(I) released from them [11], or because of the attachment of Ag(I) to the surface of the cells [15], but the dissolution of AgNPs was the main cause of the toxicity of these nanoparticles. The resulting Ag(I) would induce toxic effects once it enters the algae [43,47]. Inside the cells, silver induced oxidative stress [28,44]. Liu et al. [28] found that the *Poteroiochromonas malhamensis* accumulated AgNPs inside the cells, internalized by phagocytosis. These AgNPs caused ROS generation which leads to lipid peroxidation and membrane damage. On the contrary, He et al. [30] did not find any damage in the cell membrane of *Chattonella marina* in the presence of AgNPs, however, the cell size increased.

3.1.2. Ag-kaolin ecotoxicity

Algae were exposed to different concentrations of the leachates of Ag-kaolin and control kaolin (without silver). The growth rate and the inhibition of the growth rate are shown in table 4.7. The inhibition increased as the concentration of silver increased, being complete at a silver concentration of $10 \mu\text{g L}^{-1}$. The growth rate for the control kaolin was similar to that of the control algae, which means that the kaolin does not inhibit growth, but the silver released from Ag-kaolin. The effect is comparable to the effect of Ag(I)

standard (table 4.5) since silver was mainly released as Ag(I) from Ag-kaolin, as shown in Chapter 2 (page 130). In fact, no significant statistical differences were found between Ag(I) standard and Ag-kaolin.

Table 4.7. Growth rate and percentage of the growth rate inhibition for *R. subcapitata* exposed to Ag-kaolin. Mean $\pm$ standard deviation ($n=3$).

Sample	Silver added ($\mu\text{g L}^{-1}$)	Growth rate (day^{-1})	Growth rate inhibition (%)
Control kaolin	0	0.574 ± 0.048	-
Ag-kaolin	0.1	0.544 ± 0.130	5.3 ± 1.1
	1.0	0.485 ± 0.074	15.5 ± 1.8
	10	0	100
	50	0	100

The cytograms obtained are shown in figure 4.9. When the algae were exposed to a concentration of $50 \mu\text{g L}^{-1}$ of silver, a loss of fluorescence after the first 24 h of exposure was observed, which is also observed by the microscope (figure A3.2 in Appendix 3). Over time, the intensity of the chlorophyll fluorescence was decreasing and after 72 h of exposure, there was no fluorescence. This loss of fluorescence causes a reduction in cell density, which is also observed. For an equivalent concentration of $10 \mu\text{g L}^{-1}$ of silver, after 48 h of exposure, there was a displacement of the distribution to smaller values of fluorescence. For the other concentrations, there was no loss of fluorescence, however, two populations of cells appeared after 48 h of exposure, which was also observed when the algae were exposed to control kaolin. In figure 4.10, the cytograms for algae without exposition and algae after 72 h control kaolin, 0.1 and $1 \mu\text{g L}^{-1}$ of Ag-kaolin exposition are shown. In these cytograms, the formation of these two populations with different sizes is shown. The reason could be the formation of cell aggregates, which could cause growth inhibition due to the reduction of access to light and absorption of nutrients [23]. This effect was also visible for the kaolin exposition, which means that the kaolin particles also have an impact on the size of the algae, although the impact does not cause the loss of fluorescence.

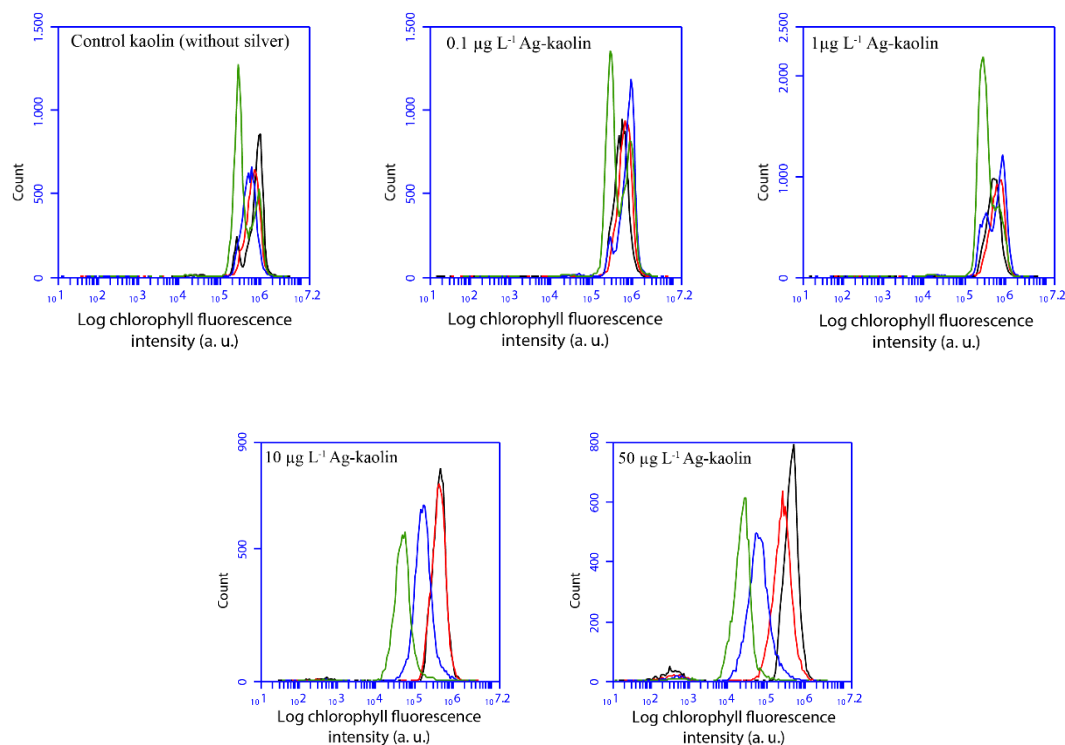


Figure 4.9. Autofluorescence cytograms for algae cells during 72 h exposed to leached Ag-kaolin. Black: 0 h. Red: 24 h. Blue: 48 h. Green: 72 h.

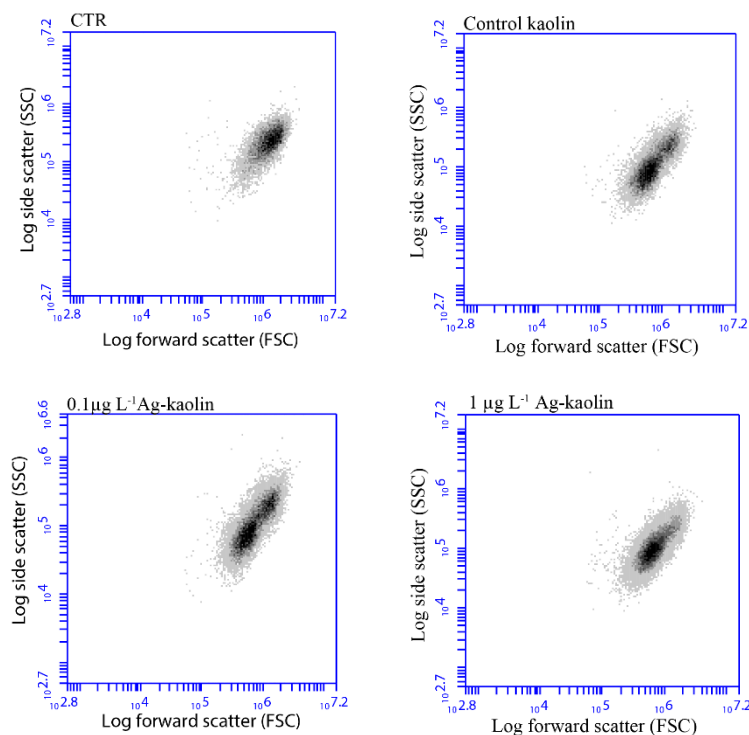


Figure 4.10. SSC/FSC dot-plot cytograms of *R. subcapitata* without exposition and exposed to control kaolin, 0.1 and 1 µg L⁻¹ of Ag-kaolin for 72 h.

Figure 4.11 shows the microscope images obtained for the different concentrations after 72 h of exposition. With the increase of the concentration of silver, the density of cells visualized decreased, as the chlorophyll fluorescence. In addition, the shape of the cells changed with the exposition to Ag-kaolin, swelling and deforming itself, losing the perfect shape of “C” (figure 4.12).

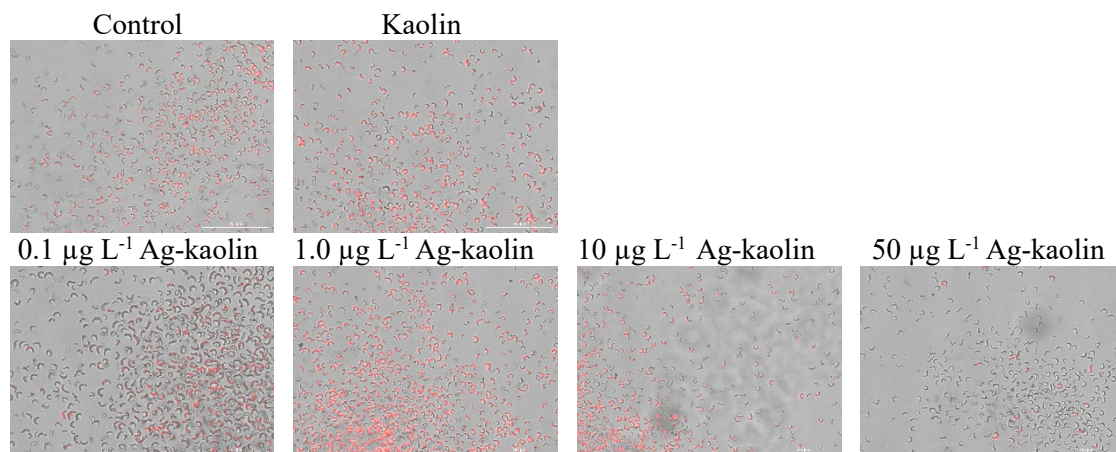


Figure 4.11. Chlorophyll fluorescence images for *R. subcapitata* after 72 h exposed to leachate of Ag-kaolin.

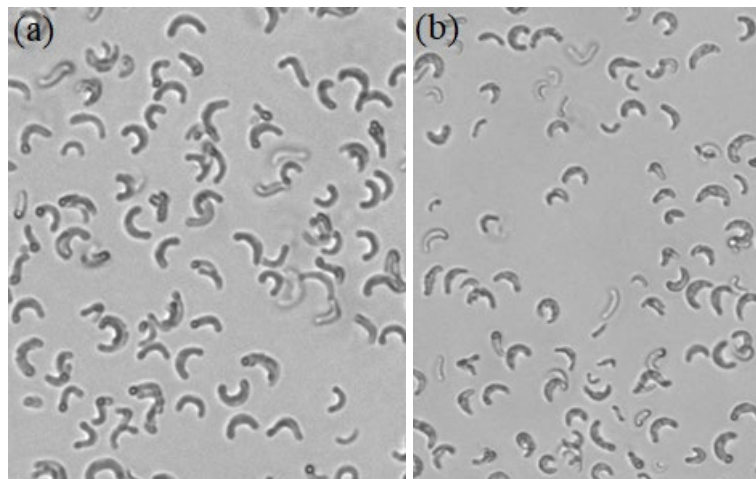


Figure 4.12. Ampliation of the algae images (a) Without exposition. (b) After 72 h exposed to $0.1 \mu\text{g L}^{-1}$ of leachate of Ag-kaolin.

3.1.3. Faeces ecotoxicity

The growth rate and the inhibition for the exposition to faeces are shown in table 4.8. For the samples of Ag20, the inhibition was higher than for Ag200. The presence of a higher

number of bacteria for the Ag20 (due to the necessity of adding a higher volume of leachate to reach the same concentration of silver) is a possible explanation for this because the theoretical concentration of silver is the same for both expositions. The growth inhibition was very low for 0.1, 1 and 50 $\mu\text{g L}^{-1}$, with an inhibition of 25% for Ag20 faeces and 7% for Ag200 faeces for the highest concentration. Nevertheless, with a concentration of 10 $\mu\text{g L}^{-1}$ of silver, the growth was higher than the growth in the control. This suggests that there would be an hormesis effect, and that concentration would be the stimulation point [48]. Hormesis is a response to contaminants which consists of showing different effects depending on the concentration of exposition [49,50]. At one point there is a stimulatory effect of the growth, the reproduction, or the chlorophyll fluorescence, which corresponds to the stimulation point. Hormesis is related to an adaptative response of the organism to protect itself from different contaminants [50]. The presence of sulphur and the organic matter of the faeces, which would make the matrix different from the other samples, could be responsible for the promotion of this effect because, in the bibliography, cysteine has been reported as the cause of this effect on human cells [48].

Table 4.8. Growth rate and percentage of growth rate inhibition for *R. subcapitata* exposed to faeces. Mean $\pm$ standard deviation ($n=3$).

Sample	Silver added ($\mu\text{g L}^{-1}$)	Growth rate (day^{-1})	Growth rate inhibition (%)
Control faeces	0	0.661 ± 0.052	-
Ag20 faeces	0.1	0.496 ± 0.034	24.9 ± 3.2
	1.0	0.548 ± 0.160	17.0 ± 5.0
	10	0.747 ± 0.019	0
	50	0.494 ± 0.018	25.3 ± 0.9
Ag200 faeces	0.1	0.601 ± 0.033	9.09 ± 0.51
	1.0	0.606 ± 0.016	8.34 ± 0.22
	10	0.718 ± 0.116	0
	50	0.677 ± 0.105	6.67 ± 1.14

Figure 4.13 shows the autofluorescence cytograms for the Ag20 faeces. As it is seen, two populations appeared, one which corresponds to algae, the right peak, and the other, at the left, which corresponds to bacteria. To discriminate between these two populations and measure the number of algae correctly it was necessary to define a gate which corresponds to algae and be able to quantify only the populations of the algae. To remove any instrument background, first, the FSC/SSC cytogram of the algae without exposition

was required (figure 4.14). The cells inside that gate were plotted in a count vs. chlorophyll fluorescence intensity plot. Another gate was applied to ensure the algae population. An example of the application of this gate with a suspension of algae exposed to $10 \mu\text{g L}^{-1}$ of Ag20 faeces is represented in figure 4.15.

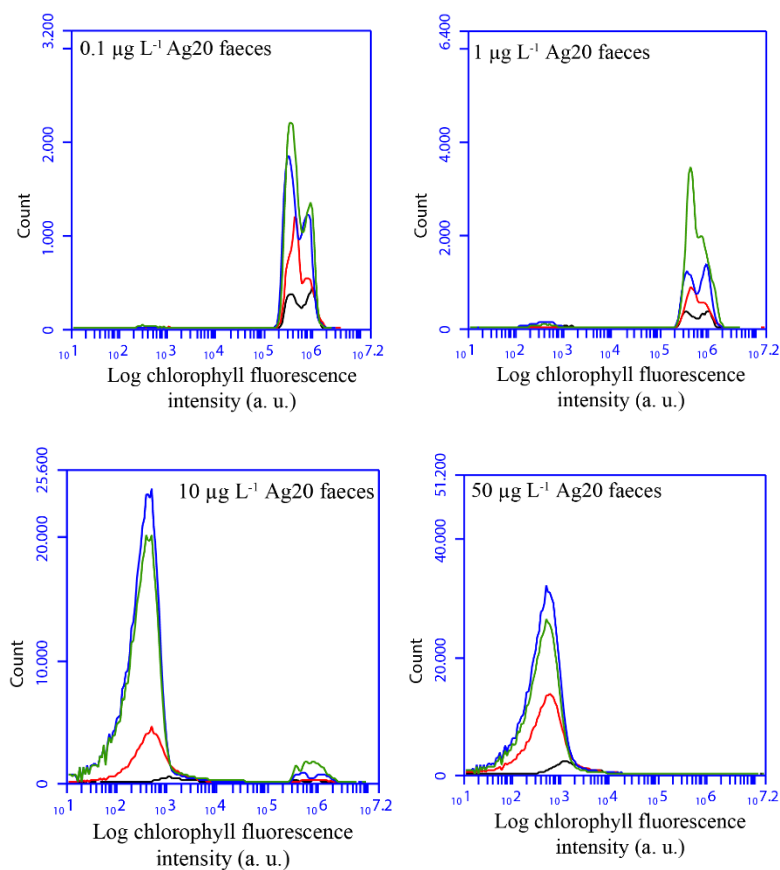


Figure 4.13. Autofluorescence cytograms for algae cells during 72 h exposed to leached Ag20 faeces. Black: 0 h. Red: 24 h. Blue: 48 h. Green: 72 h.

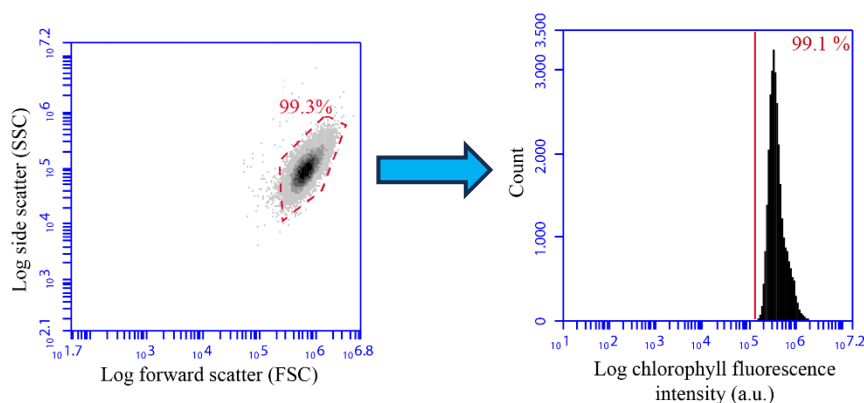


Figure 4.14. Representation of the procedure used to define the gate correspondence to algae.

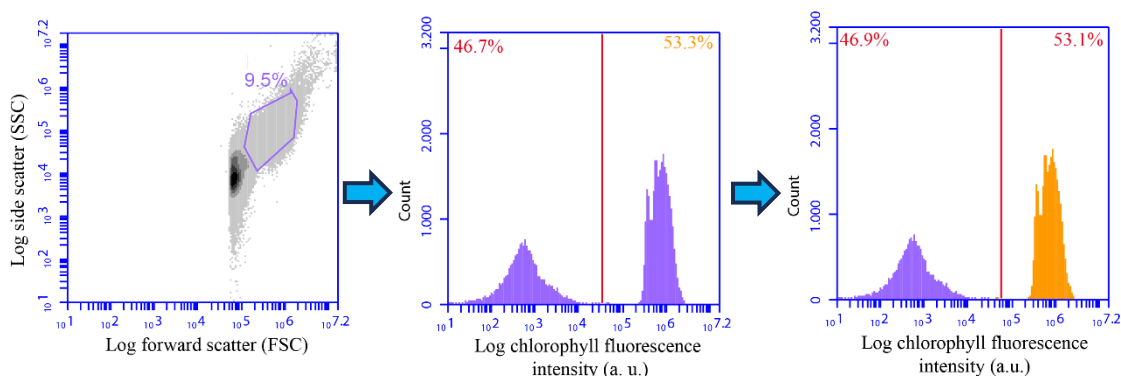


Figure 4.15. Cytograms for algae cells with exposition to the leachate of $10 \mu\text{g L}^{-1}$ of Ag20 faeces.

This gate was applied to all the samples and in figures 4.16 and 4.17 the fluorescence cytograms with the application of the gate obtained are shown. The cytograms show that there was no loss of fluorescence after 72 h of exposition, which is also visible in the microscopy images (figure 4.18 and figures A3.5 y A3.6 in Appendix 3 for the images of every 24 h of exposition of Ag20 and Ag200 faeces, respectively). However, a high number of bacteria were shown in the images, especially for $50 \mu\text{g L}^{-1}$, for both types of faeces. In addition, changes in the morphology were visualized at 10 and $50 \mu\text{g L}^{-1}$ of silver, losing the “C” shape, and showing some swelling (figure 4.19). The cell density observed in the suspension of $50 \mu\text{g L}^{-1}$ of the leachate of Ag20 faeces was lower than for the Ag200 faeces, which agrees with the results obtained for the inhibition (table 4.8).

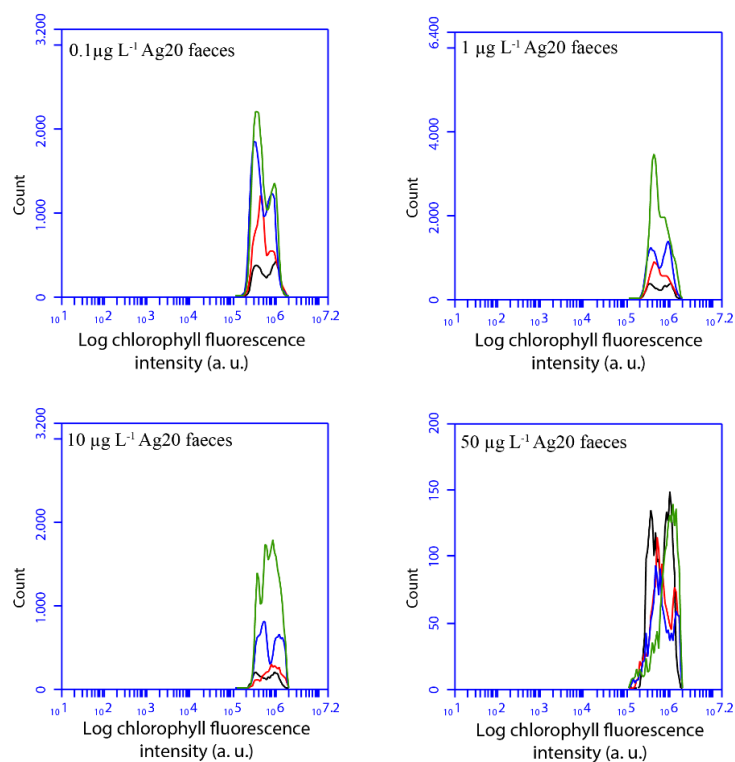


Figure 4.16. Autofluorescence cytograms with the gate applied for *R. subcapitata* during 72 h exposed to leached Ag20 faeces. Black: 0 h. Red: 24 h. Blue: 48 h. Green: 72 h.

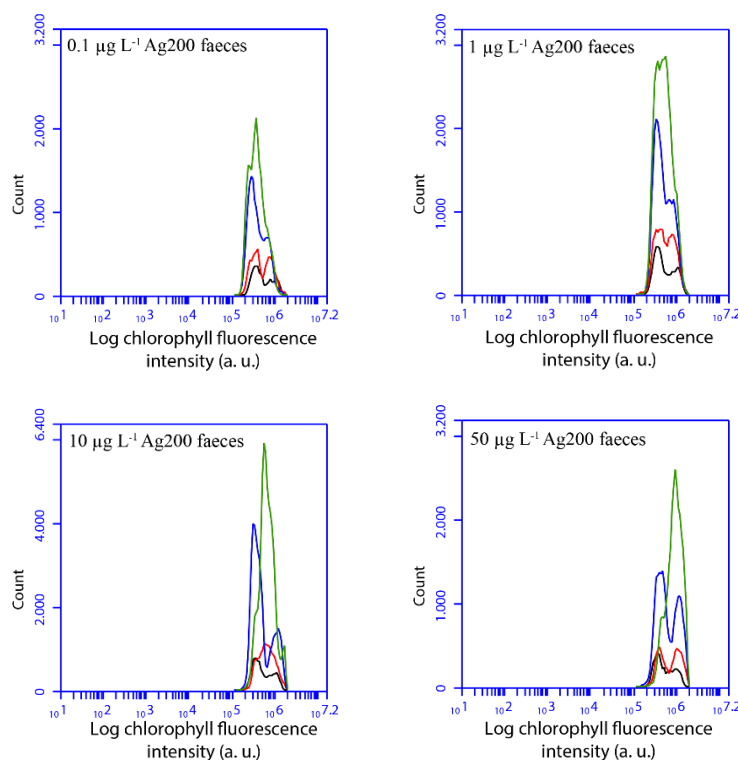


Figure 4.17. Autofluorescence cytograms with the gate applied for *R. subcapitata* during 72 h exposed to leachate of Ag200 faeces. Black: 0 h. Red: 24 h. Blue: 48 h. Green: 72 h.

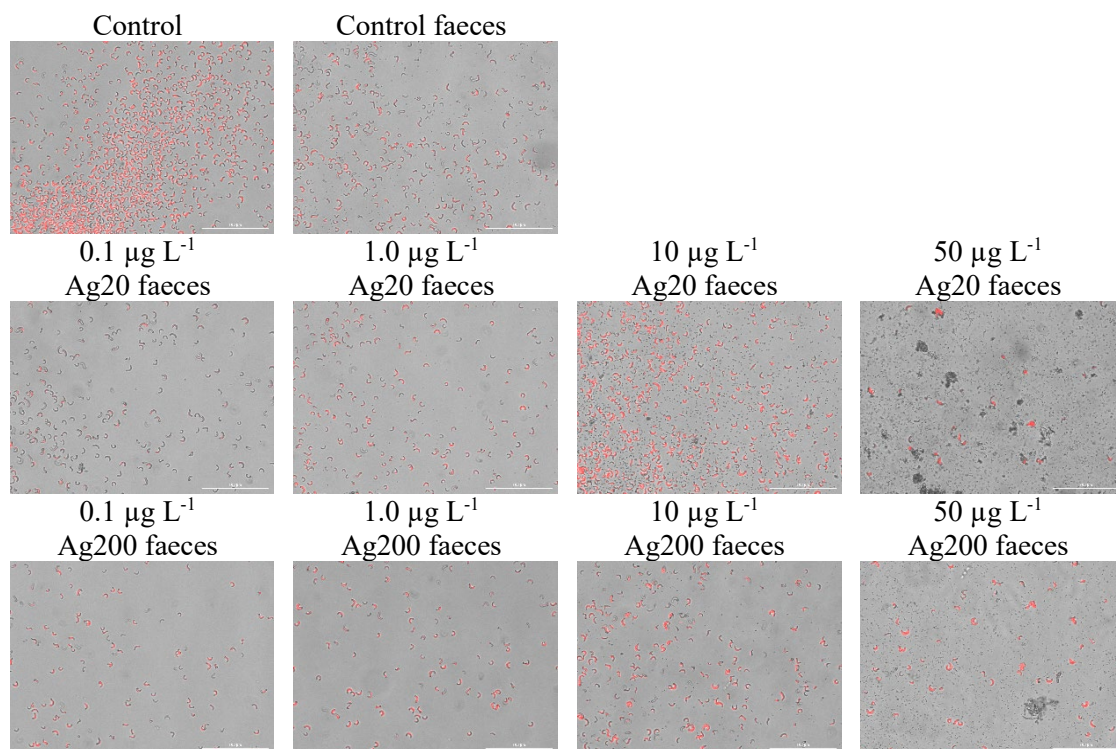


Figure 4.18. Chlorophyll fluorescence images for *R. subcapitata* after 72 h for exposition to leachate of the faeces.

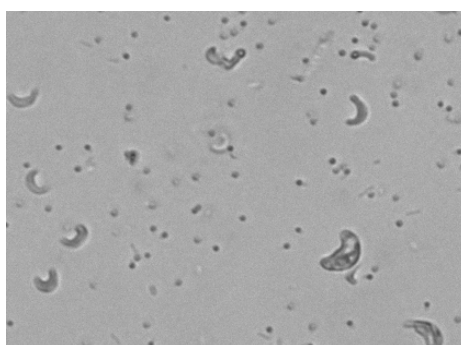


Figure 4.19. Ampliation of the algae image of the exposition to $10 \mu\text{g L}^{-1}$ of the leachate of Ag20 faeces.

The inhibition obtained was less than the obtained for the Ag-kaolin, with significant differences at the highest concentration. This is not a coincidence, because, as it was observed in the previous chapter, part of the silver released in the leachate from the faeces (Chapter 3, section 3.2.1, page 177) could be bound to sulphur. In the bibliography, it was demonstrated that Ag_2S was less toxic than Ag(I) and AgNPs [4,51]. It had been demonstrated that the formation of this silver species blocked the uptake of silver-free ions in several organisms [51–53] and its toxicity was less than that of Ag(I) . Jagadeesh

et al. [54] found that 0.1 mg L^{-1} of Ag_2S caused a percentage of toxicity (through the inhibition of the different enzyme activity) of less than 15% after 10 days of exposure. Kang et al. [51] found that Ag_2S -NPs showed lower toxicity or, even, no toxicity, in *R. subcapitata* than AgNPs. Less dissolution rate than AgNPs and lower bioavailability are the reasons for the decrease in toxicity [51]. In addition, part of the silver release was bound to organic particles and kaolin with higher sizes (section 3.2.1 in Chapter 3 (page 177)). This silver would not be internalized by algae, decreasing the toxicity of the faeces despite its total content of silver.

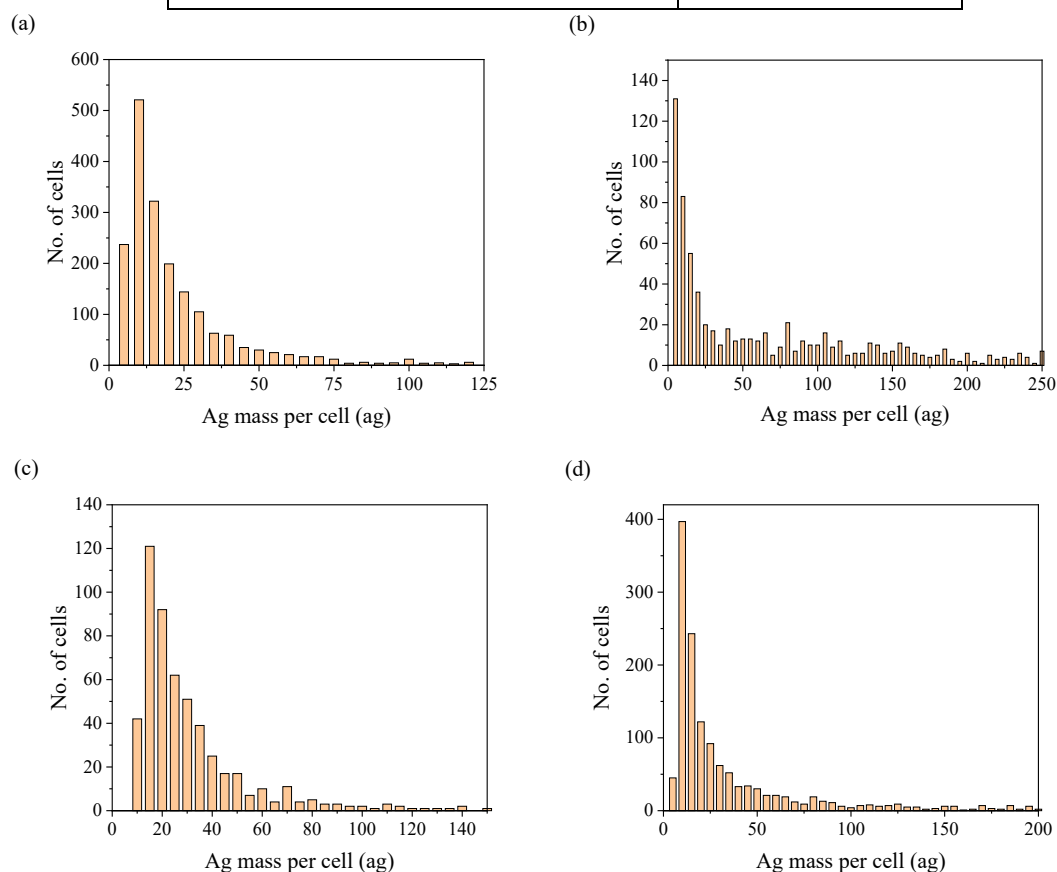
Additionally, the toxicity of the feed was studied, whose results are in Appendix 3, section A3.2. Less toxicity than that for Ag-kaolin was found. Nevertheless, it was higher than the toxicity of faeces, with $50 \text{ } \mu\text{g L}^{-1}$ of silver from feed, there was 100% growth rate inhibition. However, there was no loss of fluorescence.

3.2. Silver uptake by algae measured by SC-ICP-MS

To investigate the silver uptake, algae suspensions exposed to silver standards and samples were analysed by SC-ICP-MS. The number of events obtained for the algae without exposition (control algae) and all the samples are in table 4.9. As it is shown, the number of events collected was higher for the algae exposed than the algae without exposition, which means that there was an internalization of silver, which was confirmed by the mass distributions (figure 4.20). The most frequent silver mass per cell obtained was between 5 and 15 ag, meanwhile, for the algae without exposition, no distribution for the silver mass per cell was obtained. Ribeiro et al. [11] found, indeed, that *R. subcapitata* uptake Ag(I) , proposing that the transport of silver inside the cell occurred by accidental cation transport, with the same mechanism as the internalisation of Na^+ or K^+ . This mechanism would not be able to distinguish between Ag(I) and Cu(I) , due to their similar characteristics. Inside the cell, the Ag(I) was diffused through the cytoplasm and was bound to the thiol group of the cysteine of some proteins [55].

Table 4.9. Number of events found for the control algae and the exposition to suspensions of $10 \mu\text{g L}^{-1}$ of Ag(I) and the leachates of the samples by SC-ICP-MS.

Sample	Number of events
Control algae (without exposition)	245 ± 50
Ag(I)	1735 ± 320
Ag-kaolin	740 ± 12
Feed	795 ± 170
Ag20 faeces	1401 ± 108

**Figure 4.20.** Mass distributions obtained by SC-ICP-MS of *R. subcapitata* exposed to $10 \mu\text{g L}^{-1}$ of (a) a standard solution of Ag(I) (b) leachate of Ag-kaolin (c) leachate of feed (d) leachate of Ag20 faeces.

As it was seen before for the exposition to $10 \mu\text{g L}^{-1}$ of feed and faeces, there was no complete inhibition, seeing a possible hormesis effect in faeces, in contrast to the results obtained for Ag(I) standard and Ag-kaolin. Nevertheless, in the mass distributions (figure 4.20), the uptake of silver was observed. Thus, it can be assumed that the matrix affects, not only the internalization of the silver but also its toxicity inside the cell.

3.3. Inhibition comparison between two green algae

To compare the toxicity of the samples studied for two green algae (*R. subcapitata* and *C. reinhardtii*), the effect on the chlorophyll fluorescence was studied.

In figure 4.21, a comparison between the behaviour of the two algae in presence of Ag(I) and AgNPs standard solutions are shown. When the algae were exposed to Ag(I) (figure 4.21a), with 10 and 50 $\mu\text{g L}^{-1}$ of Ag(I) there was no inhibition of the growth for *C. reinhardtii*, contrary to what happened with *R. subcapitata*. In the case of AgNPs (figure 4.21b), although the inhibition was not complete for both algae, the effect on the growth was higher for *R. subcapitata* than for *C. reinhardtii*.

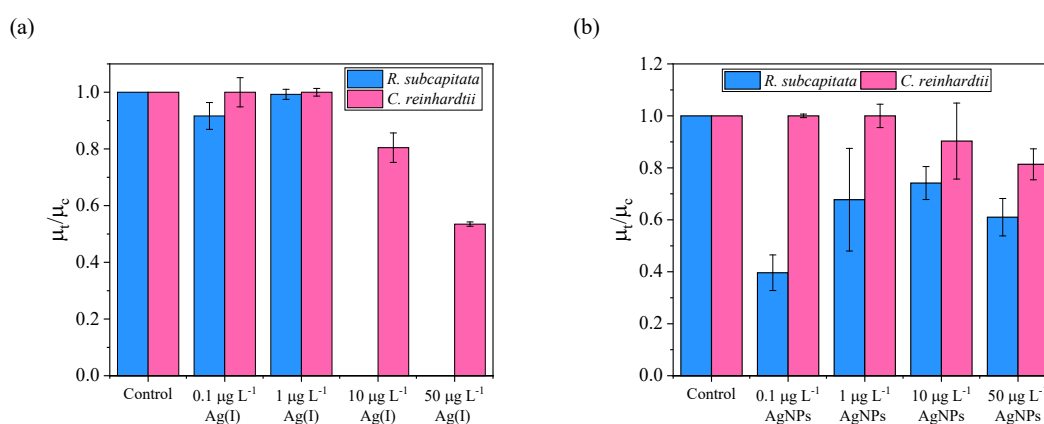


Figure 4.21. Comparison of the growth rate in terms of changes in the fluorescence in presence of different concentrations of silver standards. (a) Ag(I) (b) 50 nm AgNPs.

In figure 4.22, the results obtained for both algae for Ag-kaolin exposition are shown. For concentrations of 0.1 and 1 $\mu\text{g L}^{-1}$ of silver from the leachate of Ag-kaolin, the tendency was similar for both algae. However, for 10 and 50 $\mu\text{g L}^{-1}$, while for *R. subcapitata* there was a complete inhibition of the growth, for *C. reinhardtii*, despite there being an inhibition of the growth, the inhibition was not complete. The same tendency as the one obtained before for Ag(I), where *R. subcapitata* was more sensitive to Ag(I) than *C. reinhardtii*.

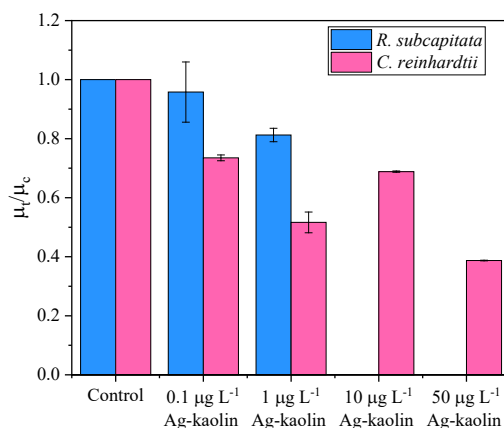


Figure 4.22. Comparison of the growth rate in terms of changes in the fluorescence in presence of different concentrations of Ag-kaolin.

In figure 4.23, the results obtained for the exposition of the feed are shown. It is seen that for 50 μg L⁻¹, for *R. subcapitata*, the inhibition was complete, meanwhile, for *C. reinhardtii*, the growth rate was higher than the growth without the sample, which means that the feed would have an hormesis effect on *C. reinhardtii*, but not on *R. subcapitata*.

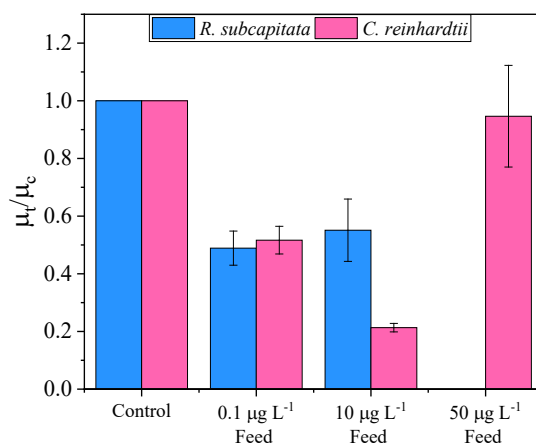


Figure 4.23. Comparison of the growth rate in terms of changes in the fluorescence in presence of different concentrations of feed.

In figure 4.24, the relations between the growth rates for the algae exposed to the faeces are shown. As in the other samples, for a concentration of 50 μg L⁻¹, there was a difference between them. For *C. reinhardtii* the inhibition was lower than the obtained for *R. subcapitata*. Moreover, the hormesis effect found with the concentration of 10 μg L⁻¹

(section 3.1.3) was not observed for *C. reinhardtii*. Still, the toxicity of faeces was less than that obtained for the other samples.

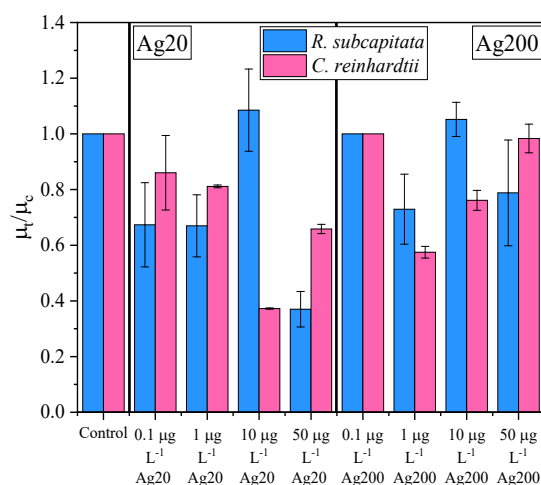


Figure 4.24. Comparison of the growth rate in terms of changes in the fluorescence in presence of different concentrations of faeces.

After 72 h, *C. reinhardtii* was visualized by microscopy. In figure 4.25, the images obtained are shown. In the images no loss of fluorescence after the exposition of any sample was shown, which agrees with the bibliography [17]. *C. reinhardtii* is less sensitive to silver, the uptake rate is higher for *R. subcapitata* than for *C. reinhardtii* which results in less toxicity [17]. Nevertheless, a difference between the algae exposed to the samples and without exposition was shown. The exposition of silver caused a stressful situation, which brought about the union of various cells, in contrast to what was observed without contaminants (figure 4.2 in section 2.4), in which the cells stayed single. This aggregation can cause the inhibition of growth due to the reduction of access to light and nutrients [23], as it was mentioned before.

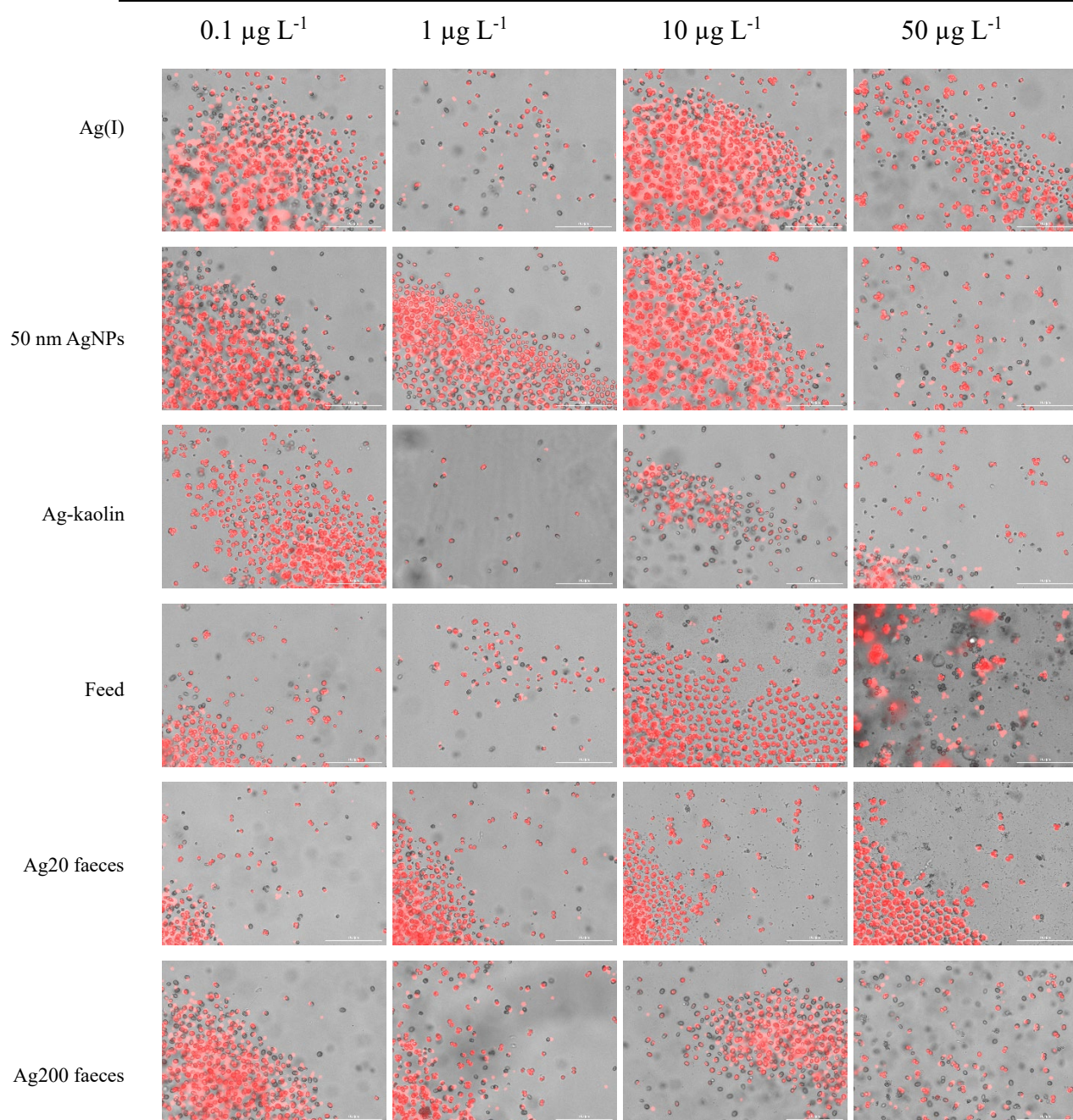


Figure 4.25. Images obtained by microscopy for *C. reinhardtii* after 72 h of exposition to the different samples.

4. Conclusions

The ecotoxicity of silver standards (Ag(I) and AgNPs) and various samples, in which Ag-kaolin nanomaterial was present (pristine Ag-kaolin and faeces from pigs fed with the supplemented feed) was studied by exposing leachates from the different samples to green alga *R. subcapitata*. It was found that 10 $\mu\text{g L}^{-1}$ of standard Ag(I) inhibited completely the algae growth after 72 h, causing the loss of chlorophyll fluorescence after the first 24 h, which resulted in cell death, whereas for AgNPs more than 100 $\mu\text{g L}^{-1}$ was required to obtain 100% of growth inhibition. Similar results to those obtained for the Ag(I) standard were obtained for Ag-kaolin leachate, in accordance with the predominant presence of Ag(I) in the leachate. Exposure to faeces leachate led to partial growth inhibition (7-25%) for a silver concentration of 50 $\mu\text{g L}^{-1}$. This means that, although the Ag-kaolin would have a high environmental impact, it would be lower for the faeces, due to the low inhibition found in the experiments. In any case, it was confirmed the cell internalization of silver in all the exposure experiments, even if the toxicity was low. Additionally, a comparison between two green algae (*R. subcapitata* and *C. reinhardtii*) was performed, confirming that *C. reinhardtii* was less sensitive to silver. The presence in the faeces of low solubility species of silver, such as Ag₂S, and the matrix complexity, induced less toxicity to algae than ionic silver, which would result in a low environmental impact of the Ag-kaolin nanomaterial when used as an additive in pig-feeding.

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IV. CONCLUSIONS

IV. Conclusiones

Teniendo en cuenta que el objetivo principal de esta Tesis Doctoral es la evaluación de un nanomaterial base de plata (Ag-caolín) como aditivo alimentario para cerdos, haciendo uso de una plataforma de métodos analíticos que permita obtener información diferenciada de distintas formas de plata, los resultados obtenidos han permitido extraer las siguientes conclusiones:

- La cromatografía hidrodinámica acoplada a ICP-MS ha permitido separar, caracterizar en tamaño y cuantificar correctamente especies particuladas y disueltas de plata y oro. Para ello se ha desarrollado una nueva metodología basada en el uso de una fase móvil con penicilamina (0.05 mM en el caso del oro y 1 mM para plata), que ha permitido la recuperación cuantitativa de las especies iónicas, sin afectar a las especies nanoparticuladas, separándolas en menos de 10 min, con resoluciones entre 0.7 y 0.9 para las especies iónicas y nanopartículas de 10 nm. Esta metodología desarrollada ha permitido complementar la información dada por SP-ICP-MS y otras técnicas utilizadas a lo largo de esta Tesis Doctoral, especialmente para muestras con contenidos altos en especies disueltas.
- El estudio del comportamiento del nanomaterial Ag-caolín en agua ha permitido comprobar que la mayor parte de la plata liberada (menos del 40%), lo hacía en forma de Ag(I) y que el 0.01% lo hacía en forma de partículas. Por otro lado, en presencia del pienso, la plata también se liberaba mayoritariamente en forma de Ag(I), aunque en menor proporción (6%).
- La bioaccesibilidad de la plata presente en el pienso suplementado con el nanomaterial Ag-caolín, estudiada mediante simulación *in vitro* de la digestión gastrointestinal porcina ha sido de alrededor de 8%, encontrándose mayoritariamente en formas solubles de Ag(I) (99.9%). El estudio del comportamiento de especies de plata (Ag(I) and AgNPs) durante la digestión *in vitro*, ha puesto de manifiesto que la Ag(I) era altamente bioaccesible, aunque esta bioaccesibilidad disminuía significativamente en presencia de la matriz de pienso. En el caso de las nanopartículas de plata, se ha observado la oxidación parcial de las mismas, que ha sido completa en presencia de pienso.

- La baja bioaccesibilidad de la plata en el pienso se ha traducido en una baja retención de plata en los tejidos animales, tal y como se ha comprobado a partir de los análisis de muestras de músculo, hígado, riñón, heces y purines procedentes de un ensayo *in vivo* realizado con cerdos alimentados con pienso suplementado con el nanomaterial Ag-caolín. Se ha observado que la plata absorbida no se acumulaba en el músculo, pero sí lo hacía en el hígado y, en menor medida, en los riñones. La mayor parte de la plata se eliminó en heces y purines.
- La extracción secuencial basada en TSPP, amoníaco y Na₂S ha permitido fraccionar parte de la plata presente en heces en formas solubles de Ag(I), partículas de plata, de cloruro de plata y de sulfuro de plata, siendo las fracciones más abundantes las correspondientes a sulfuro de plata (45%) y plata soluble (34%). Sin embargo, no se han obtenido rendimientos cuantitativos debido al bajo rendimiento de extracción obtenido (23%). De la misma forma, para la extracción alcalina con TMAH tampoco se han obtenido rendimientos cuantitativos, por lo que su uso ha quedado restringido a confirmar la presencia de formas particuladas de plata en las heces.
- El estudio de la movilización de la plata presente en heces con agua ha permitido comprobar que menos del 3% de la plata se liberaba, mayoritariamente en forma de complejos disueltos con materia orgánica frente a otras formas particuladas.
- La exposición de una microalga verde (*R. subcapitata*) a lixiviados del nanomaterial Ag-caolín, pienso suplementado con el nanomaterial y heces de cerdos alimentados con el pienso suplementado ha permitido evaluar su ecotoxicidad y el impacto ambiental del uso del nanomaterial como aditivo alimentario. Debido a las formas poco solubles en las que se encontraba la plata en heces y su baja movilización, la ecotoxicidad de estas ha sido mucho menor que la del nanomaterial Ag-caolín o la del pienso suplementado, por lo que el impacto medioambiental del uso del aditivo Ag-caolín debería de ser bajo.

IV. Conclusions

Considering that the main objective of this Doctoral Thesis is the assessment of a nanomaterial based on silver (Ag-kaolin) as a feed additive for pigs, using a platform of analytical methods that allow to obtain information about the different forms of silver, the results obtained have led to the following conclusions:

- The hydrodynamic chromatography coupled to ICP-MS has allowed the proper separation, size characterization and quantification of the particulate and dissolved species of silver and gold. For this purpose, a new methodology based on the use of a mobile phase with penicillamine (0.05 mM for gold and 1 mM for silver) has been developed, which has allowed the quantitative recovery of ionic species, without affecting NPs, in less than 10 min and with resolutions between 0.7 and 0.9 for the ionic species and 10 nm nanoparticles. This developed methodology has led to the completion of the information given by SP-ICP-MS and other techniques used in this Doctoral Thesis, especially for samples with high contents of dissolved species.
- The study of the behaviour of the nanomaterial Ag-kaolin in water showed that most of the silver released (less than 40%), was as Ag(I) and 0.01% was as particles. On the other hand, in the presence of the feed, silver was also released mostly as Ag(I), although in a smaller proportion (6%).
- The bioaccessibility of silver present in the feed supplemented with the nanomaterial Ag-kaolin, studied by the simulation of a porcine *in vitro* gastrointestinal digestion has been around 8%, mostly as soluble Ag(I) (99.9%). The study of the behaviour of the silver species (Ag(I) and AgNPs) along the *in vitro* digestion, showed that Ag(I) was highly bioaccessible, although this bioaccessibility decreased significantly in the presence of the feed matrix. In the case of silver nanoparticles, partial oxidation has been observed, which has been complete in the presence of feed.
- The low bioaccessibility of silver in the feed resulted in low retention of silver in the animal tissues, as was shown in the analysis of the samples of muscles, liver, kidney, faeces and slurries, from an *in vivo* experiment carried out with pigs fed with supplemented feed. The absorbed silver has been observed to not accumulate

in the muscle, but in the liver and, to a lesser extent, in the kidney. Most of the silver was removed in faeces and slurries.

- The sequential extraction based on TSPP, ammonium and Na₂S has allowed the fractionation of part of the silver present in faeces in soluble forms of Ag(I), silver particles, silver chloride and silver sulphide, being silver sulphide (45%) and soluble silver (34%) the most abundant species. Nevertheless, no quantitative efficiencies have been obtained due to the low extraction efficiency obtained (23%). Similarly, with the alkaline extraction with TMAH, no quantitative efficiencies have been obtained either, so its use has been restricted to the confirmation of the presence of particulate forms of silver in faeces.
- The study of the leaching of the faeces in water has shown that less than 3% of the silver was released, mostly as dissolved complexes with organic matter against other particulate forms.
- The exposition of a green microalga (*R. subcapitata*) to leachate of the nanomaterial Ag-kaolin, feed supplemented with the nanomaterial and faeces from pigs fed with feed supplemented has allowed the assessment of their ecotoxicity and the environmental impact of the use of the nanomaterial as a feed additive. Due to the poorly soluble forms in which the silver was present in faeces and their low mobilization, their ecotoxicity has been much lower than the ecotoxicity of the nanomaterial Ag-kaolin or the supplemented feed, so the environmental impact of the use of the additive Ag-kaolin should be low.

V. APPENDIX

Appendix 1. Equations for the calculation of the limits of detection by SP-ICP-MS

To obtain the mass, size and number limit of detection, the article of Laborda et al. [1] was followed.

The equations (A.1) and (A.2) show the mass and size limits of detection, respectively; where σ_B is the blank deviation, w is the peak width, K_{ICP-MS} is the detection efficiency, which is the ratio of the ions detected versus the atoms introduced in the instrument, K_M ($=AN_{AV}/M_M$) and is related to the element measured, t_{dwell} is the dwell time, ρ the density of the element and F_R is the mass fraction of the element.

$$LOD_{mass} = \frac{5 \sigma_B}{\frac{2}{w} K_{ICP-MS} K_M t_{dwell}} \quad (A.1)$$

$$LOD_{size} = \left(\frac{30 \sigma_B}{\frac{2}{w} \pi \rho F_R K_{ICP-MS} K_M t_{dwell}} \right)^{1/3} \quad (A.2)$$

For the calculation of the number concentration limit of detection (LOD_{number}) the equation (A.3) was followed, where $\sigma_{N,B}$ is the standard deviation of the number of counted events, η is the nebulization efficiency, Q_{sam} is the sample flow rate and t_i is the total acquisition time.

$$LOD_{number} = \frac{5 \sigma_{N,B} + 3}{\eta Q_{sam} t_i} \quad (A.3)$$

References

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Appendix 2. Study of the behaviour of a silver-based nanomaterial used for animal feeding: solubility, dissolution/degradation and *in vitro* digestion

A2.1. Ag-kaolin characterization

The nanomaterial Ag-kaolin characterization was published previously by Rodriguez-Garraus et al. [1].

Ag-kaolin was provided by Laboratorios Enosán S.L. AgNPs were adsorbed into the surface of the microparticles of the kaolin. It was analysed by FESEM to determine the diameter of the particles added to the kaolin. In figure A2.1, FESEM images are shown. The brightness spots are the nanoparticles. It can be seen that the shape of the particles is spherical.

The size of the particles was measured by the software Image J. The distribution obtained is in figure A2.2. The most frequent diameters obtained were between 30-40 nm.

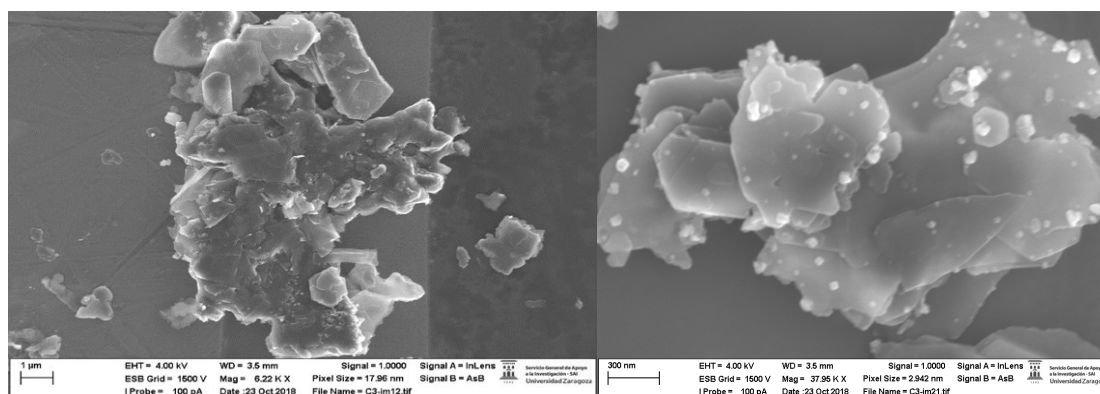


Figure A2.1. FESEM images for Ag-kaolin.

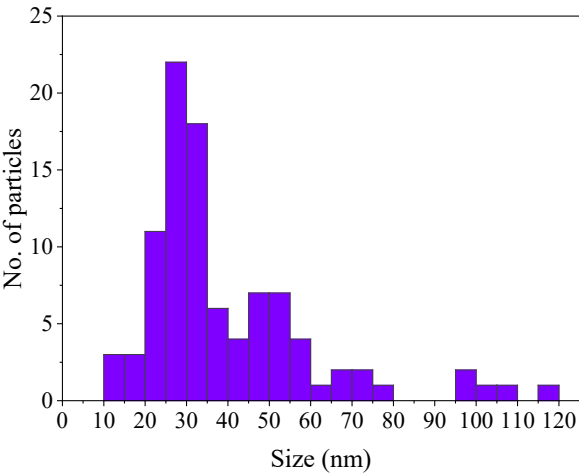


Figure A2.2. Size distribution for the silver particles of Ag-kaolin.

In table A2.1, complementary physicochemical properties of Ag-kaolin are shown.

Table A2.1. Properties of Ag-kaolin.

Parameter	Value
Silver content	8.32 ± 0.35 mg g ⁻¹
pH (*)	5.78 ± 0.16
Zeta potential (*)	-36.3 ± 0.5 mV

(*) 1 g additive L⁻¹ in ultrapure water

A2.2. Solubilization and dissolution/degradation of the feed containing Ag-kaolin in water

A2.2.1. Procedure for the study of the solubility and degree of dissolution of the feed

To determine the amount of silver in pig feed that was solubilized in water, feed suspensions with a ratio of Ag-kaolin and ultrapure water of 0.5 and 1 g L⁻¹ were prepared, weighing 0.025 and 0.05 g of feed, respectively in 50 mL with ultrapure water.

For 1 h and 24 h, the samples were shaken at 99 rpm in dark and at room temperature. Then, the suspensions were centrifuged at 7000 g for 17 min, to leave particles larger than

1 μm at the bottom of the tube. Subsequently, the supernatant and the solid were separated and the supernatant was diluted with ultrapure water to reach the optimum concentration for the analysis. ICP-MS was used to obtain the total concentration of silver released in water. SP-ICP-MS were used to obtain the different species originated from the dissolution/degradation of feed.

For the analysis by ICP-MS, a calibration curve of Ag(I) in 1% of HNO_3 with concentrations between 0 and 15 $\mu\text{g L}^{-1}$ was measured. A solution of 10 $\mu\text{g L}^{-1}$ of Rh was used as an internal standard.

For the analysis by SP-ICP-MS, firstly the sample flow rate and the transport efficiency were obtained following the procedure described in the Procedures section in Chapter 2 (page 129). For the quantification of silver, a calibration curve of Ag(I) in 1% HNO_3 with concentrations between 0 to 1 $\mu\text{g L}^{-1}$ was used.

A2.2.2. Results for the study of the solubility and degree of dissolution of the feed

The total silver concentration obtained for feed solubilization is shown in table A2.2. In general, between 4.6 and 6.6% of the total silver present in the feed was solubilized. There are no significant differences between the two incubation times, which would show that all the silver that is solubilized, mostly does so in the first hour of incubation. There are also no significant differences in the rate of solubilization between both concentrations of feed leaching so the ratio of silver solubilized does not depend on the amount of feed used.

Table A2.2. Total silver concentration released for the solubilization of pig feed in water by ICP-MS. Mean $\pm$ standard deviation ($n=3$).

Feed	Ratio solid to liquid	Incubation time	Silver released concentration (mg kg^{-1})	Silver solubilized (%)
Control	1 g L^{-1}	1 h	0.070 ± 0.016	-
		24 h	0.090 ± 0.004	-
Supplemented feed	0.5 g L^{-1}	1 h	0.698 ± 0.087	5.63 ± 0.87
		24 h	0.825 ± 0.104	6.66 ± 0.87
	1 g L^{-1}	1 h	0.580 ± 0.047	4.65 ± 0.38
		24 h	0.780 ± 0.002	6.28 ± 0.03

For the determination and quantification of the silver particles and Ag(I) concentration present during feed solubilization, the supernatants of feed suspensions in ultrapure water were also analysed by SP-ICP-MS. The amount of Ag(I) and silver particles released is shown in table A2.3. It is observed that for both incubation times and both concentrations, similar percentages were obtained, similar to the results obtained by ICP-MS (table A2.2), which confirms that practically all the silver that is solubilized does in the first hour from being in contact with water. Additionally, it is also observed that around 0.02% of the total silver released was as particles for both incubation times, which means that practically all the particles dissolved when they were solubilized. Regarding the most frequent and equivalent size of the particles, it was around 30 nm, similar to that found in the leaching of Ag-kaolin (Chapter 1, page 130). The distributions are very similar to each other. Figure A2.3 shows two size distributions for a feed suspension in ultrapure water with an incubation time of 1 h and 24 h. As for Ag-kaolin, the distributions shown are not complete due to the high concentration of dissolved and the low concentration of AgNPs. The LOD_{size} obtained is, for an incubation time of 1 h, 29 nm, which caused that part of the distribution (the part of the smallest particles) was not seen and, therefore, the rate of particles obtained was lower than the real one. For the distribution with an incubation time of 24 h, the LOD_{size} is 27 nm, similar to the limit found for 1 h of incubation.

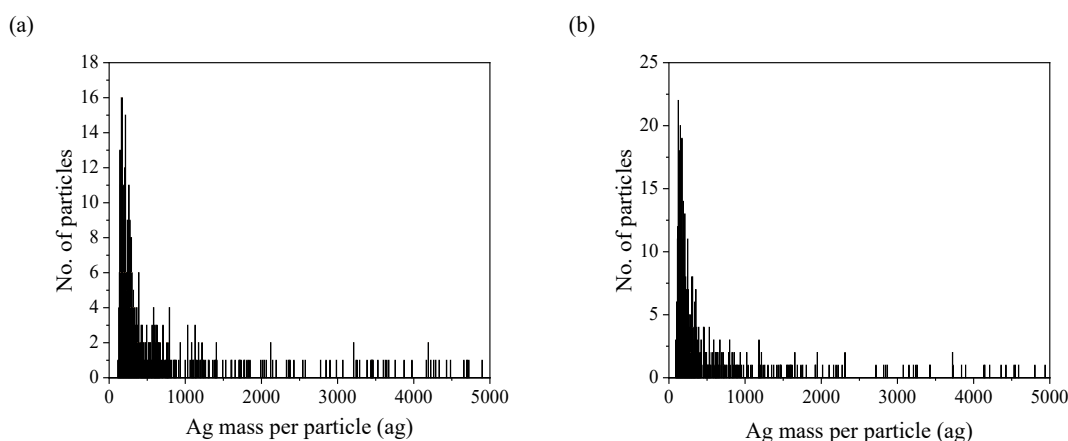


Figure A2.3. Silver mass per particle distributions for the leaching of feed supplement with Ag-kaolin in water. (a) Incubation time: 1 h (b) Incubation time: 24 h.

It is observed that, in the feed, the concentration of silver that was released was lower (around 4-6%) than the silver released from the nanomaterial alone (greater than 30%),

confirming the idea that the behaviour of the nanomaterial is different in the presence of the feed matrix and the absence of it. For this reason, it is necessary, not only to study the nanomaterial alone but also to study it within the corresponding food matrix. Regarding dissolution, in both materials, most of the silver was released as Ag(I), and less than 1% was as particles, so most of the released particles were dissolved.

Table A2.3. *Ag(I) and silver particle concentrations obtained for the solubilization of supplemented feed in water by SP-ICP-MS. Mean $\pm$ standard deviation ($n=3$).*

Ratio solid:liquid of feed	Incubation time	Ag(I) released (mg kg ⁻¹)	Ag(I) released vs. total silver (%)	Most frequent equivalent size (nm)	AgNPs released (µg kg ⁻¹)	AgNPs released vs. total silver (%)
0.5 g L ⁻¹	1 h	0.521 $\pm$ 0.054	4.21 $\pm$ 0.44	36 $\pm$ 7	2.28 $\pm$ 0.27	0.018 $\pm$ 0.002
	24 h	0.631 $\pm$ 0.006	5.09 $\pm$ 0.05	34 $\pm$ 2	1.75 $\pm$ 0.34	0.014 $\pm$ 0.003
1 g L ⁻¹	1 h	0.236 $\pm$ 0.044	4.11 $\pm$ 0.35	36 $\pm$ 2	2.13 $\pm$ 0.21	0.017 $\pm$ 0.002
	24 h	0.234 $\pm$ 0.169	4.94 $\pm$ 1.36	32 $\pm$ 2	1.50 $\pm$ 0.07	0.012 $\pm$ 0.001

References

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Appendix 3. Ecotoxicity of Ag-kaolin, feed and pig faeces to green algae

A3.1. Time course of the effects in *R. subcapitata*

The images obtained for the chlorophyll fluorescence every 24 h until 72 h of exposure to solutions of Ag(I) and AgNPs standards are shown in figure A3.1 and A3.2.

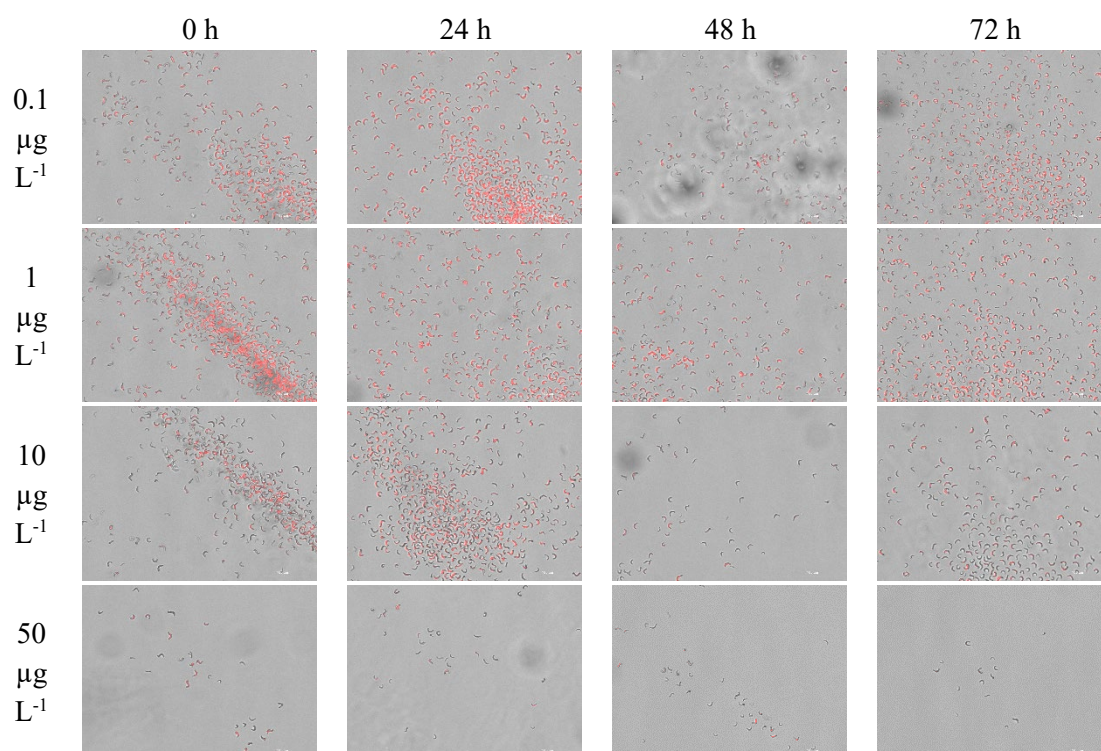


Figure A3.1. Images obtained by microscope for the exposition of the algae to different concentrations of Ag(I) standard solutions every 24 h.

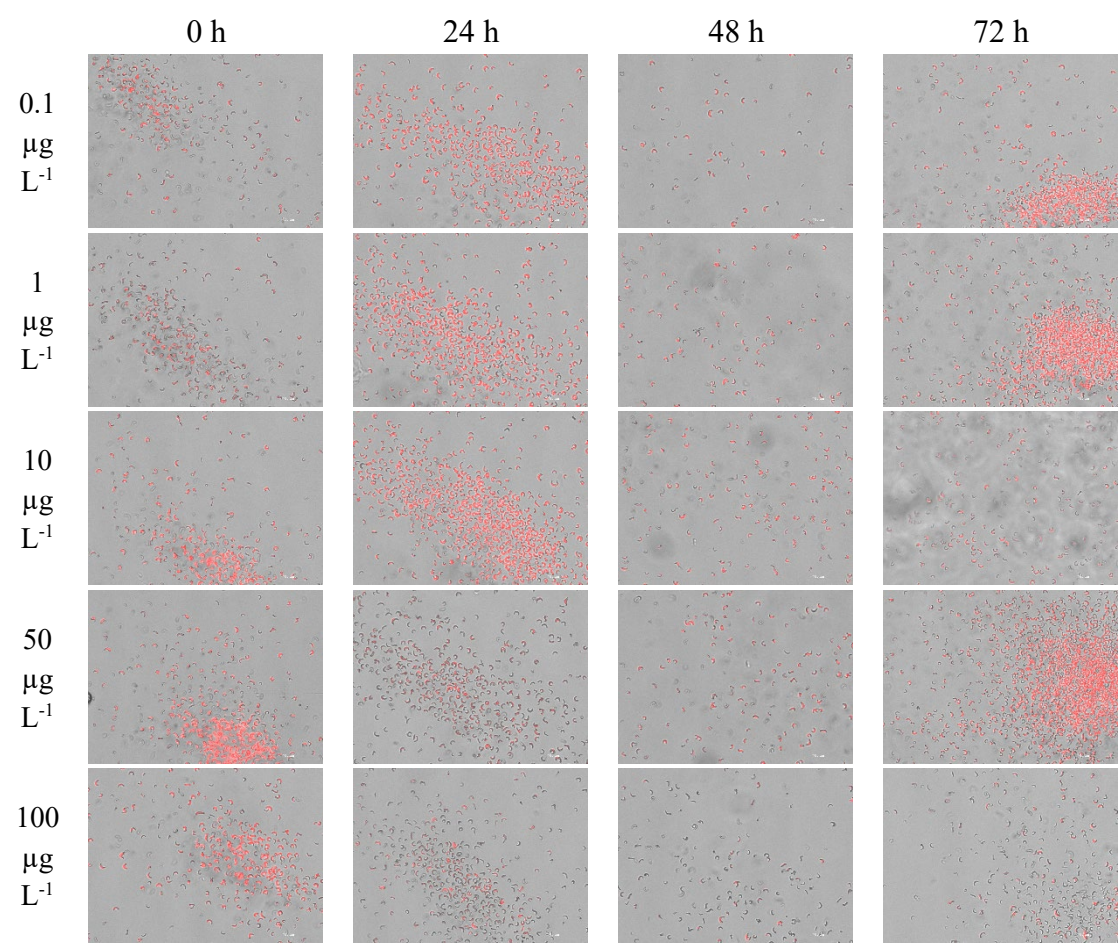


Figure A3.2. Images obtained by microscope for the exposition of the algae to different concentrations of 50 nm AgNPs standard solutions every 24 h.

The images obtained by the microscope for the different samples of silver are in figures A3.3 (Ag-kaolin), A3.4 (feed), A3.5 and A3.6 (Ag 20 and Ag200 faeces, respectively).

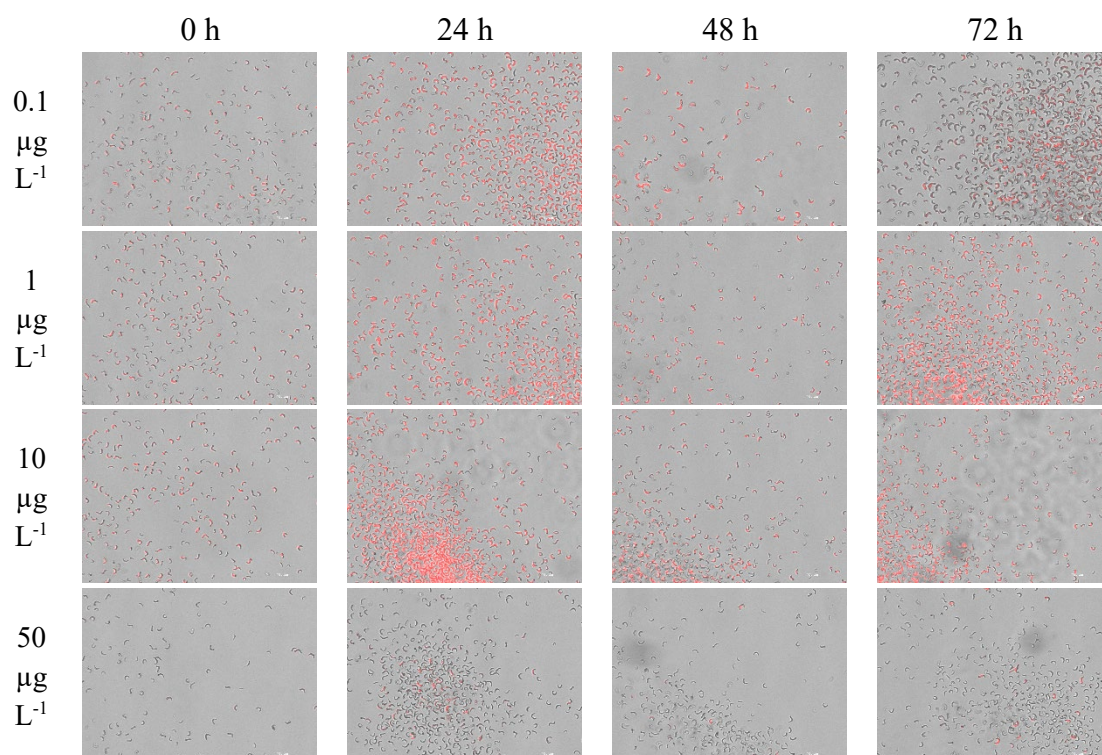


Figure A3.3. Images obtained by microscope for the exposition of the algae to different concentrations of leachates of Ag-kaolin every 24 h.

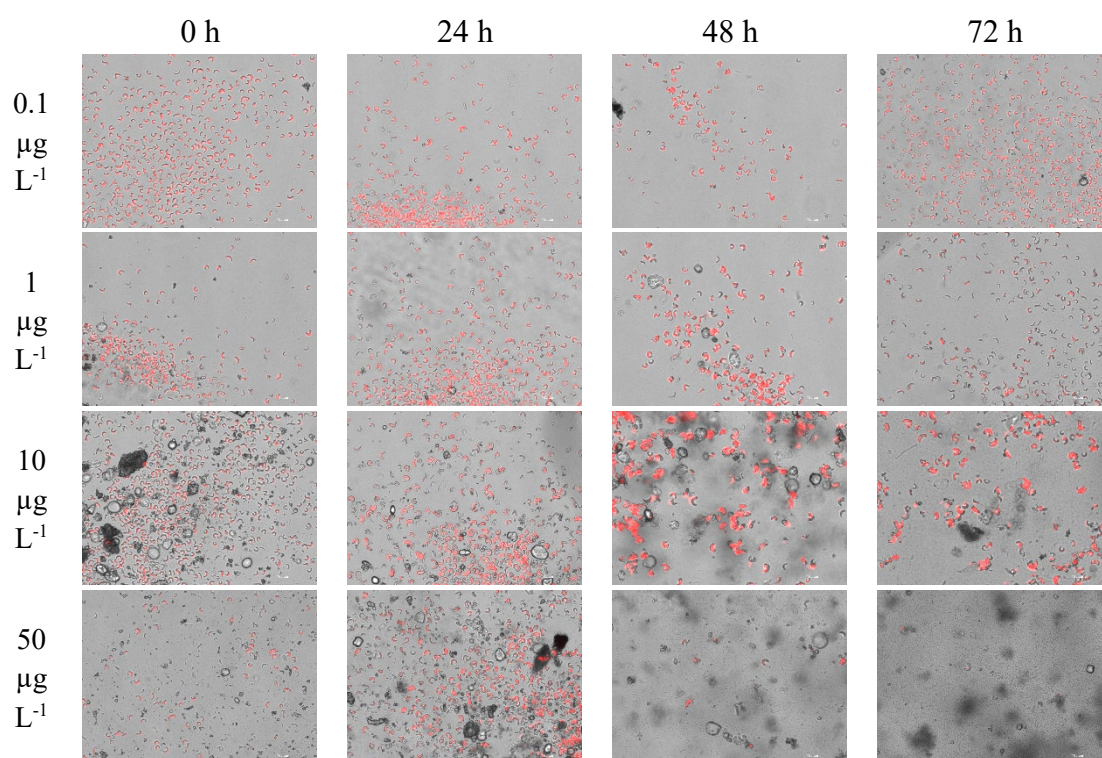


Figure A3.4. Images obtained by microscope for the exposition of the algae to different concentrations of leachates of feed every 24 h.

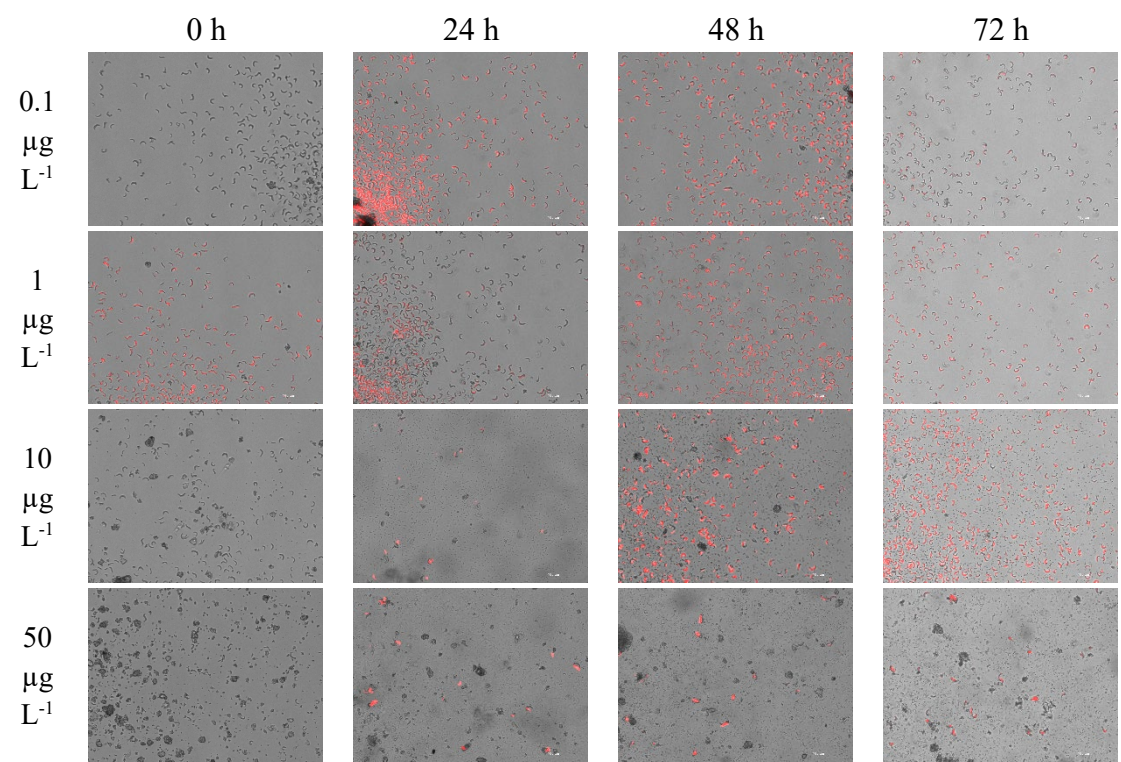


Figure A3.5. Images obtained by microscope for the exposition of the algae to different concentrations of leachates of Ag20 faeces every 24 h.

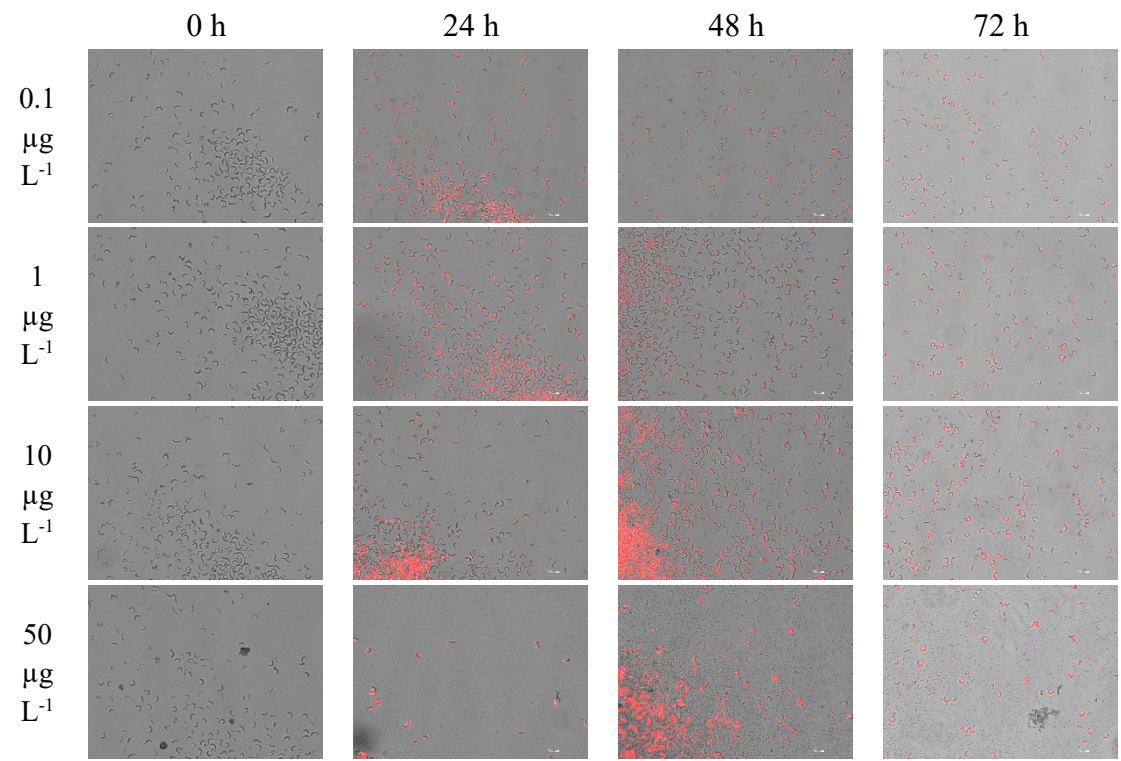


Figure A3.6. Images obtained by microscope for the exposition of the algae to different concentrations of leachates of Ag200 faeces every 24 h.

A3.2. Effect of different silver-containing materials on the algal growth

A.3.2.1. Feed supplemented with Ag-kaolin ecotoxicity

R. subcapitata was exposed to the leachate of the feed supplement with Ag-kaolin. The results for the growth rate and the inhibition are shown in table A3.1. In contrast to the results obtained for Ag-kaolin, with a concentration of $10 \mu\text{g L}^{-1}$ the inhibition of the growth was not complete, but at $50 \mu\text{g L}^{-1}$, the inhibition was 100%. No significant differences were found for the behaviour at the highest concentration with the Ag(I) and Ag-kaolin, nevertheless, for the rest of the concentrations, significant differences were found by ANOVA.

Table A3.1. Growth rate and percentage of the growth rate inhibition for *R. subcapitata* exposed to supplemented feed. Mean $\pm$ standard deviation ($n=3$).

Sample	Silver added ($\mu\text{g L}^{-1}$)	Growth rate (day^{-1})	Growth rate inhibition (%)
Control feed	0	0.547 ± 0.129	-
Feed	0.1	0.526 ± 0.058	3.84 ± 0.42
	1.0	0.425 ± 0.174	22.4 ± 0.2
	10	0.371 ± 0.008	32.2 ± 0.6
	50	0	100

The results of the chlorophyll fluorescence obtained every 24 h by flow cytometry are shown in figure A3.7. Two populations appeared in the cytograms, one which corresponds to algae, the right peak, and the other, at the left, which corresponds to bacteria. To measure the number of algae correctly it was necessary to apply a gate to these cytograms and only see the populations of the algae. The procedure described in Chapter 4 (section 3.1.3, page 230) was followed. As an example, in figure A3.8, the procedure done for the exposition to $10 \mu\text{g L}^{-1}$ is shown.

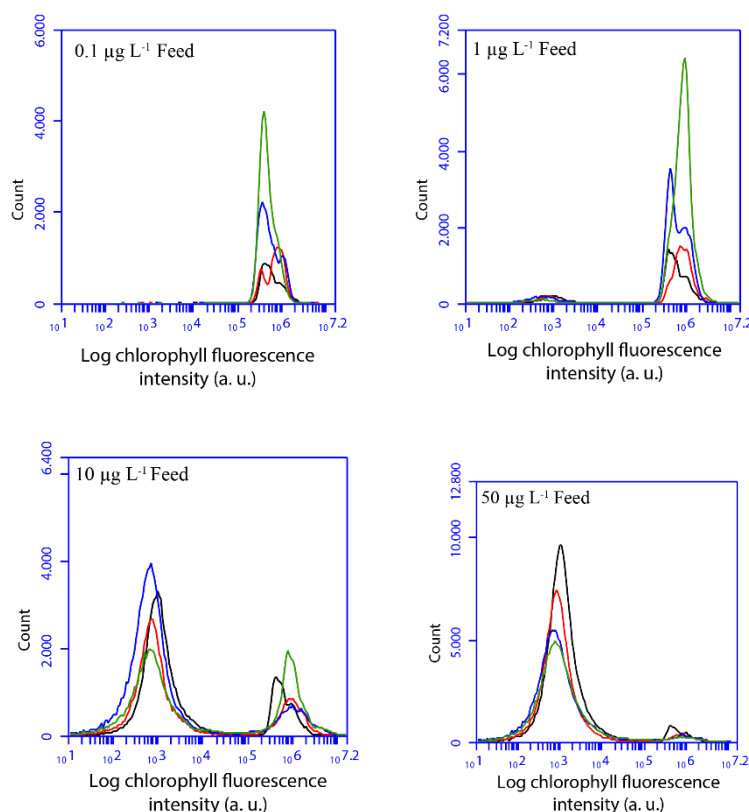


Figure A3.7. Autofluorescence cytograms for *R. subcapitata* during 72 h exposed to leached feed. Black: 0 h. Red: 24 h. Blue: 48 h. Green: 72 h.

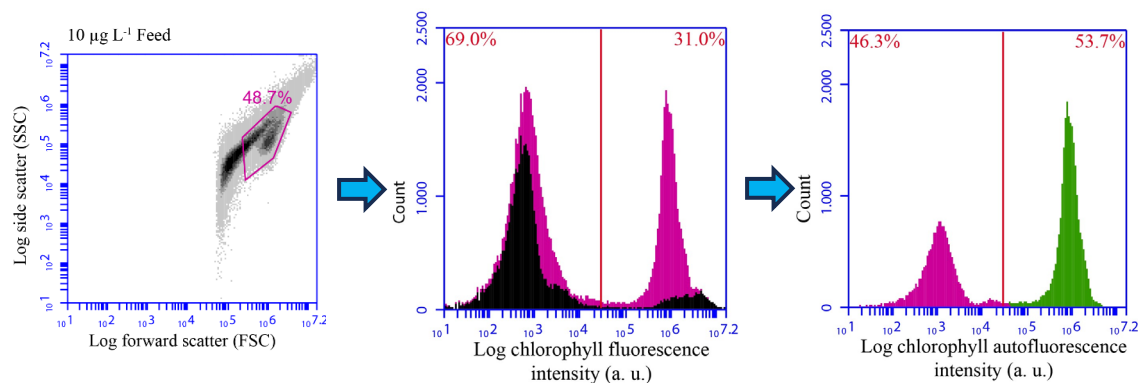


Figure A3.8. Cytograms for *R. subcapitata* with exposition to the leachate of $10 \mu\text{g L}^{-1}$ of feed.

Fluorescence cytograms obtained with the gate applied are shown in figure A3.9. No loss of fluorescence was observed, unlike the results obtained for Ag(I), AgNPs and Ag-kaolin. So, it can be concluded that the matrix affects the toxicity of the samples. The bacteria visualized in the microscope images (figure A3.4) confirm the identification of

the left peak in the autofluorescence cytograms as bacteria. Also, in the images, the cell density that was visualized, decreased for the exposition to $50 \mu\text{g L}^{-1}$. The high number of bacteria made it difficult to see the algae cells. In addition, for the concentrations of 10 and $50 \mu\text{g L}^{-1}$, it was shown since the first 24 h (figure A3.5), changes in the morphology of the cells, as swelling. Nevertheless, for the concentration of $50 \mu\text{g L}^{-1}$, the high number of bacteria from the feed and the inhibition of the growth, made impossible the detection of the cells.

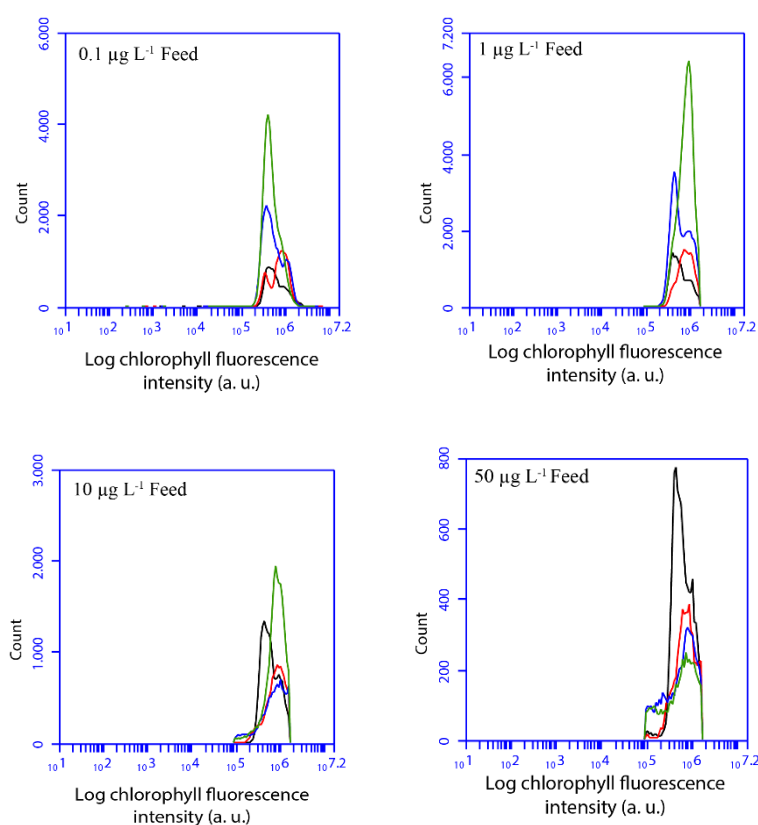


Figure A3.9. Autofluorescence cytograms with the gate applied for *R. subcapitata* for 72 h exposure to leached feed. Black: 0 h. Red: 24 h. Blue: 48 h. Green: 72 h.

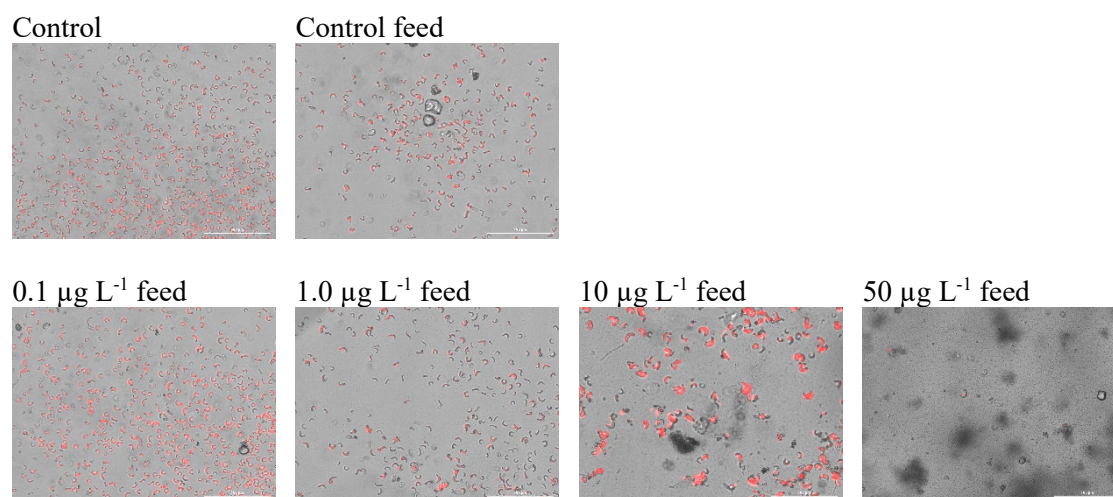


Figure A3.10. Chlorophyll fluorescence images for *R. subcapitata* after 72 h exposed to leachate of feed.

Appendix 4. Publications

- M.S. Jiménez, M. Bakir, D. Isábal, M.T. Gómez, J. Pérez-Arantegui, J.R. Castillo, F. Laborda, Evaluation of hydrodynamic chromatography coupled to inductively coupled plasma mass spectrometry for speciation of dissolved and nanoparticulate gold and silver, *Anal. Bioanal. Chem.* 413 (2021) 1689–1699.
<https://doi.org/10.1007/s00216-020-03132-3>.
- E. Bolea, M.S. Jimenez, J. Perez-Arantegui, J.C. Vidal, M. Bakir, K. Ben-Jeddou, A.C. Gimenez-Ingalaturre, D. Ojeda, C. Trujillo, F. Laborda, Analytical applications of single particle inductively coupled plasma mass spectrometry: a comprehensive and critical review, *Anal. Methods*. 13 (2021) 2742–2795.
<https://doi.org/10.1039/D1AY00761K>.
- M. Bakir, K. Ben-Jeddou, A.C. Gimenez-Ingalaturre, E. Bolea, F. Laborda, Single Particle Inductively Coupled Plasma Mass Spectrometry: A Valuable Tool in Environmental Analysis, in: *Environmental Nanopollutants*, The Royal Society of Chemistry, 2022: pp. 321–338.
<https://doi.org/10.1039/9781839166570-00321>
- M.S. Jiménez, M. Bakir, K. Ben-Jeddou, E. Bolea, J. Pérez-Arantegui, F. Laborda, Comparative study of extraction methods of silver species from faeces of animals fed with silver-based nanomaterials, *Microchim. Acta*. 190 (2023) 204.
<https://doi.org/10.1007/s00604-023-05777-0>.
- K. Ben-Jeddou, M. Bakir, M.S. Jiménez, M.T. Gómez, F. Laborda. Nanosilver-based materials as feed additives: Evaluation of their transformations along in vitro gastrointestinal digestions in pigs and chickens by using an ICP-MS based analytical platform. *Food Chem.* Pending acceptance.



Evaluation of hydrodynamic chromatography coupled to inductively coupled plasma mass spectrometry for speciation of dissolved and nanoparticulate gold and silver

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Abstract

In this study, hydrodynamic chromatography coupled to inductively coupled plasma mass spectrometry has been evaluated for the simultaneous determination of dissolved and nanoparticulate species of gold and silver. Optimization of mobile phase was carried out with special attention to the column recovery of the different species and the resolution between them. Addition of 0.05 mM penicillamine to the mobile phase allowed the quantitative recovery of ionic gold and gold nanoparticles up to 50 nm, whereas 1 mM penicillamine was necessary for quantitative recovery of ionic silver and silver nanoparticles up to 40 nm. The resolution achieved between ionic gold and 10-nm gold nanoparticles was 0.7, whereas it ranged between 0.31 and 0.93 for ionic silver and 10-nm silver nanoparticles, depending on the composition of mobile phase. Best-case mass concentration detection limits for gold and silver species were 0.05 and 0.75 $\mu\text{g L}^{-1}$, respectively. The developed methods allowed the simultaneous detection of nanoparticulate and dissolved species of gold and silver in less than 10 min. Size determination and quantification of gold and silver species were carried out in different dietary supplements, showing good agreement with the results obtained by electron microscopy and total and ultrafiltrable contents, respectively. Due to the attainable resolution, the quality of the quantitative results is affected by the relative abundance of nanoparticulate and dissolved species of the element and the size of the nanoparticles if present.

Keywords Gold · Silver · Nanoparticles · Speciation · Hydrodynamic chromatography, ICP-MS

Introduction

The continuous advances in nanoscience involve the growing use of different engineered nanoparticles (ENPs) in an increasing number of consumer products. This fact requires the adaptation of existing techniques and methods, or the development of new ones, to monitor their occurrence, fate, and

transformations in different scenarios. In order to understand the environmental impact or the toxicological mechanisms of inorganic ENPs, it is critical to discriminate among dissolved and particulate forms of the element involved. Different techniques and methodological approaches for the characterization and quantification of ENPs and its derivatives in complex samples have been recently reviewed [1–5].

Asymmetric flow field flow fractionation (AF4) and hydrodynamic chromatography (HDC) are commonly coupled to inductively coupled plasma mass spectrometry (ICP-MS) as an element-specific detector for the separation and determination of inorganic ENPs in a variety of samples [1, 3]. Gray et al. [6] compared HDC and AF4, both coupled with ICP-MS, with respect to their capacity to detect, quantify, and characterize gold nanoparticles (AuNPs). They found that, although HDC is a robust and versatile separation technique, its resolving power is much lower than AF4, being even lower in the smallest size range. On the other hand, recoveries for HDC were better than for AF4 on average, ranging from 77 to

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In memory of Juan R. Castillo who passed away a few days before the publication of this article.

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96% for HDC and from 4 to 89% for AF4. An additional advantage of HDC over AF4 lies in the analysis time, which can be reduced to less than 10 min, in comparison with 30–45 min of AF4, and dissolved low molecular mass species are not lost, as in AF4 due to the ultrafiltration membranes used in its separation channel. Thus, HDC-ICP-MS can provide simultaneous information about dissolved and particulate species of an element in less than about 10 min, which is not the case of AF4.

HDC is a liquid chromatographic separation technique in which the sample is injected into a column packed with non-porous beads, building up flow channels, and separation is produced by the velocity gradient within the capillaries between beads. Thus, larger particles are transported faster than smaller ones, as they spend less time near the edges of the capillaries. In packed columns, the beads should be inert, that is, made of a material that minimizes non-HDC enthalpic interactions between the beads and the dissolved analytes. Such non-HDC effects can be minimized through the addition of salts and/or surfactants to the solvent/mobile phase to screen electrostatic and van der Waals interactions, which are especially common in aqueous media [7]. Separation in HDC arises from the parabolic or Poiseuille-like flow profile that develops, under laminar flow conditions in the interstitial medium of a packed column, where the fastest streamlines of flow are in the middle of the interstitial medium and the slowest ones are near the packing particles. The first experimental paper on HDC was published in 1974 by Small [8], who employed a series of packed columns to separate and determine the particle size of various polystyrene latexes, carbon black, and colloidal silica. Since the first application of HDC coupled to ICP-MS [9], HDC has become popular in environmental analysis to understand the behavior, occurrence, and fate of nanomaterials (NMs) and colloids [10–14]. Due to the simplicity of the separation mechanism, the size determination and method development are straightforward [7, 9, 11]. For instance, the coupling of HDC to ICP-MS in single-particle mode (SP-ICP-MS), first described by Pergantis et al. [15], was applied to characterize AuNPs in drinking water by determining particle mass, hydrodynamic diameter, and number concentration.

The relative low size resolution of HDC compared to other size separation methods [5] is more relevant when analyzing NP mixtures [6, 9, 15, 16]. Gray et al. [6] showed the inability of HDC to separate a mixture of AuNPs, but also showed potential for separation of the Au dissolved from the AuNP standards. They suggested it might be possible to separate Au dissolved from NPs greater than 20 nm although without reporting any recoveries for dissolved Au. To the best of our knowledge, there are very few references in which the separation and potential quantification of ENPs and their corresponding metal ions using HDC-ICP-MS have been studied. Philippe et al. [11] studied the effect of the eluent composition

on the retention factors of polystyrene nanoparticle standard, Rh^{3+} and AuNPs. They reported that when using a mobile phase without phosphate ions, the recovery for gold ions was 20% worse than when phosphate is used due probably to a stabilization effect of phosphate ions throughout the elution. In any case, they did not report any quantitative recoveries for AuNPs neither for ionic Au. Roman et al. [17] using HDC-SP-ICP-MS determined the concentration of dissolved Ag and the distribution of silver NPs (AgNPs), in terms of hydrodynamic diameter, mass-derived diameter, and number and mass concentration, in plasma and blood of burn patients. Peak from dissolved Ag in plasma (most probably complexed by proteins) could not be chromatographically resolved from those of 20-nm particles; thus, an innovative algorithm was implemented to deconvolute the signals of dissolved Ag and AgNPs and to extrapolate a multiparametric characterization of the particles in the same chromatogram. Pitkänen et al. [18] studied the quantitative characterization of AuNPs contrasting size-exclusion chromatography (SEC) and HDC coupled with ICP-MS. They obtained that column recoveries for AuNPs were complete and at least matched for both alternative methods. They reported a recovery value of $99 \pm 2\%$ for ionic Au using HDC-ICP-MS although no chromatogram of AuNPs and ionic Au all together was shown.

The aim of this work was to develop and evaluate separation methods based on the use of hydrodynamic chromatography coupled to ICP-MS for the simultaneous speciation of dissolved species and nanoparticles of gold and silver, the references being scarce in bibliography. The aim is focused in both nanoparticle size characterization and quantification of the different species. Optimization of the proposed HDC-ICP-MS method was carried out paying special attention on column recovery and resolution between dissolved species and nanoparticles. The methods were applied to the size characterization and quantitation of gold and silver species in dietary supplements containing nanoparticulate and/or ionic species.

Experimental

Materials and methods

Instrumentation

The high-performance chromatographic system used was a Waters 2796 Bioseparations Module (Waters Corporation, Milford, USA). HDC separations were performed in a PL-PSDA type 1 column (Agilent Technologies, Germany) with a nominal separation range of 5–300 nm, a length of 80 cm, and an internal diameter of 7.5 mm. The exit of the column was connected in series to an UV-visible detector (Waters 996 photodiode array detector) and to an ICP mass spectrometer (ELAN DRC-e, PerkinElmer, Toronto, Canada) for element-

specific detection. The outflow from the system was delivered directly to the nebulizer of the spectrometer, and a glass concentric slurry nebulizer with a cyclonic spray chamber (Glass Expansion, Melbourne, Australia) was used. Table 1 summarizes the experimental ICP-MS and HDC conditions. Origin 8 (OriginLab, Northampton, MA, USA) was used for processing the chromatograms.

A gold dietary supplement was analyzed by transmission electron microscopy (TEM) with a JEOL-2000 FXII (JEOL Ltd., Tokyo, Japan) at 200 kV. The sample was prepared by placing 20 μL of the supplement suspension on a carbon-coated copper grid and drying at room temperature.

Silver dietary supplements were analyzed by field emission scanning electron microscopy (FESEM) with energy-dispersive spectrometry (EDS). The observations were performed in a MerlinTM FESEM microscope equipped with a Gemini column (Carl Zeiss Nano Technology Systems, Germany), working at 5 kV with the in-Lens secondary-electron detector, in order to improve resolution. FESEM was coupled with an X-Max X-ray microanalyzer (Oxford Instruments, UK). Twenty microliters of the product was deposited on a copper grid holder and, once the solvent was evaporated, coated with carbon. Image analysis was carried out using ImageJ software, and average particle diameter was determined by randomly measuring the area of a certain number of particles in the images, and calculated as equivalent circle diameter.

Chemicals

Diluted suspensions of gold and silver nanoparticles were prepared from commercially available suspensions. Suspensions of gold nanoparticles, stabilized in 2 mM citrate with nominal concentrations of 50 mg L^{-1} and nominal diameters of 10, 50, and 100 nm (11.6 ± 1 , 52

± 6 , and 97 ± 11 nm, respectively), and suspensions of silver nanospheres (NanoXact), stabilized in 2 mM citrate with nominal concentrations of 20 mg L^{-1} and nominal diameters of 10, 20, 40, and 60 nm (10.3 ± 2.1 , 18.6 ± 2.7 , 39 ± 5 , and 59 ± 6 nm), were all purchased from NanoComposix (San Diego, CA, USA). Ionic gold (1001 ± 4 mg L^{-1}) and ionic silver (994 ± 3 mg L^{-1}) standards were provided by Fluka BioChemika (Buchs, Switzerland).

Table 2 summarizes the different mobile phases tested for HDC separations. The pH of the mobile phases was in all cases 7.5. Na_2HPO_4 , DL-penicillamine (PA), and Triton X-100 were obtained from Sigma-Aldrich Chemie (Stenheim, Germany); sodium dodecyl sulfate (SDS) from Bio-Rad Laboratories (Hercules, USA); and formaldehyde 37% (w/w) solution from Probus (Barcelona, Spain).

Ultrapure water (Milli-Q Advantage, Molsheim, France) was used for the preparation of the HDC mobile phases and dilutions.

Samples

A liquid nutritional supplement containing gold nanoparticles, with a nominal concentration of 20 mg L^{-1} and a nominal size of 3.2 nm, was purchased from a website distributor.

Three commercial colloidal silver products, purchased from different website distributors, were also analyzed. These products are recommended as health products intended for oral administration or as surface sanitizers for external use. The products were kept in a dark place at room temperature until analysis.

Determination of the total Au and Ag concentration in nanoparticle suspensions and nutritional supplements

Dissolution of gold nanoparticles was performed by adding 150 μL of nitric acid (69/70%, J.T. Baker (Phillipsburg, USA)) and 310 μL of hydrochloric acid (37%, J.T. Baker (Phillipsburg, USA)) to 100 μL of the suspension to be analyzed [11]. After 1 h at room temperature, solutions were diluted with ultrapure water up to 10 mL. The concentrations of total gold in AuNPs and the nutritional supplement were measured by ICP-MS using rhodium as the internal standard. Dissolution of AgNPs was performed by adding 750 μL of nitric acid to 750 μL of AgNP standard, and after 1 h at room temperature, solutions were diluted with ultrapure water up to 5 mL. For the nutritional supplements, between 500 μL and 1.5 mL of nitric acid were added to 500 μL of the supplement, after 1 h at room temperature, solutions were diluted with ultrapure water up to 5 mL. The concentrations of total silver in AgNPs and nutritional supplements were measured by flame atomic absorption spectroscopy (FAAS).

Table 1 Instrumental operating conditions

ICP-MS	
Forward power (W)	1100
Argon gas flow rate	
Plasma (L min^{-1})	15
Auxiliar (L min^{-1})	1.2
Nebulizer gas flow rate (L min^{-1})	1.0
Sweeps per reading	1
Dwell time (ms)	50
Acquisition mode	Peak hopping
Isotopes monitored	^{196}Au , ^{107}Ag , ^{109}Ag
HDC	
Column	PL-PSDA type 1
Flow rate (mL min^{-1})	1.6
Injection volume (μL)	50

Table 2 Composition of the different mobile phases used in HDC-ICP-MS separations

Gold species	
• 0.5 mM Na ₂ HPO ₄ , 0.45 mM SDS, 0.05% Triton X-100, 0.05% formaldehyde (CM)	pH 7.5
• 0.45 mM SDS	pH 7.5
• 0.45 mM SDS, 0.05–0.5 mM DL-penicillamine	pH 7.5
Silver species	
• 0.5 mM Na ₂ HPO ₄ , 0.45 mM SDS, 0.05% Triton X-100, 0.05% formaldehyde, 0.05–1 mM DL-penicillamine	pH 7.5
• 0.22–0.45 mM SDS, 1 mM DL-penicillamine	pH 7.5

Determination of ionic gold and silver in nutritional supplements

The ionic species in the suspensions were isolated by removing gold and silver nanoparticles using Nanosep Pall centrifugal ultrafilter devices with cut-off membranes of 3 kDa (equivalent to 2-nm hydrodynamic diameter). Ultrafiltration devices were washed by centrifugation with 500 μ L of ultrapure water twice. The second washing was kept to check for any potential contamination. Suspensions were sonicated for 2 min, and 500 μ L was subjected to centrifugation for 30 min at 9000 rpm and 20 °C (in a Thermo Heraeus Multifuge X1R, equipped with a fixed angle rotor for Eppendorf tubes, Waltham, USA). For gold determination, the ultrafiltrate (ca. 500 μ L) was diluted up to 10 mL with 5% HCl prior to ICP-MS analysis. For silver determination, the ultrafiltrate (ca. 500 μ L) was diluted up to 5 mL with 1% HNO₃ prior to FAAS analysis. The recovery of ionic gold and silver from the ultrafiltration devices under the operating conditions described above was better than 90% for both elements.

Column recovery calculation

Potential losses of analytes during the chromatographic separations were evaluated through the corresponding column recoveries, which were calculated from the ratio of the ICP-MS peak areas of standards injected into the chromatographic system with and without the HDC column, under the selected operational conditions. Three replicates of each measurement were performed. In total, 250 ng mL⁻¹ of Au(III), AuNPs (10, 50, and 100 nm), Ag(I), and AgNPs (10, 20, 40, and 60 nm) solutions were used for recovery calculations. Au(III) and Ag(I) solutions were stabilized with different concentrations of PA depending on the mobile phase used.

Resolution calculation

In all cases, resolution was calculated as $2(t_2 - t_1)/(w_1 + w_2)$, where t_1 and t_2 are retention times, and w_1 and w_2 are peak widths of 10-nm AuNPs or AgNPs and ionic Au or Ag, respectively.

Results and discussion

Selection of mobile phases

Although different mobile phases have been reported for the separation of metallic nanoparticles by HDC, the column manufacturer recommends a mobile phase consisting of 0.5 mM Na₂HPO₄, 0.45 mM SDS, 0.05% Triton X-100, 0.05% formaldehyde, and pH 7.5 (CM mobile phase), which is used in most of the published works as such or with small modifications [6, 9, 11, 12, 17]. The low ionic strength of this mobile phase reduces electrostatic interactions between the NPs and the packing material because of the increase of the double layer [19]; the non-ionic surfactant Triton X-100 is used to prevent the aggregation of nanoparticles, whereas the anionic surfactant SDS prevents the sorption of nanoparticles onto the column packing material. Formaldehyde is used for its bactericidal properties and sodium hydrogen phosphate helps to maintain the neutral pH of the mobile phase.

Whereas most of works have focused on the separation of nanoparticles, the objective of this work was the development of chromatographic methods that allowed the separation of dissolved forms of gold and silver from their nanoparticle counterparts with adequate resolution and recoveries of the different species. Figure 1a shows the separation of AuNPs using the manufacturer mobile phase at a flow rate of 1.6 mL min⁻¹. AuNPs standards of 10, 50, and 100 nm were eluted at 8.26, 8.08, and 7.94 min, respectively. Recoveries for the AuNPs ranged from 68 to 77% (Table 3), whereas ionic gold was fully retained in the column.

By using a simpler mobile phase containing 0.45 mM SDS at pH 7.5 (based on our previous experience [20]), AuNPs were eluted at slightly different retention times (8.10, 7.90, and 7.82 min for 10-, 50-, and 100-nm AuNPs, respectively), as it can be shown in the chromatogram of Fig. 1b. Better recoveries were obtained for AuNPs of 10 and 50 nm, except for 100 nm (88, 83, and 44%, respectively, see Table 3), although ionic gold was still retained into the column and no peak was obtained.

Although the nature of the packing material is not available from the manufacturer, cation exchange resin beads have been reported as a typical packing in HDC [21]. Thus, it seems

Table 3 Recoveries for gold and silver species separated by HDC-ICP-MS and different mobile phases ($n = 3$). Flow rate, 1.6 mL min^{-1} ; CM, mobile phase recommended by the column manufacturer. Mean $\pm$ standard deviation ($n = 3$)

Mobile phase	Recovery (%)					
	Au(III)	10-nm AuNPs	50-nm AuNPs	100-nm AuNPs		
CM	Not eluted	77 ± 1	74 ± 1	69 ± 3		
0.45 mM SDS	Not eluted	88 ± 1	83 ± 5	44 ± 2		
0.45 mM SDS +						
0.05 mM PA	94 ± 3	103 ± 1	102 ± 2	72 ± 9		
0.20 mM PA	88 ± 5	114 ± 2	82 ± 2	66 ± 4		
0.50 mM PA	89 ± 6	116 ± 2	76 ± 8	61 ± 2		
	Ag(I)	10-nm AgNPs	20-nm AgNPs	40-nm AgNPs	60-nm AgNPs	
CM +						
0.05 mM PA	25 ± 4	66 ± 5	66 ± 4	66 ± 9	24 ± 1	
0.2 mM PA	40 ± 1	100 ± 5	107 ± 11	90 ± 9	49 ± 3	
0.5 mM PA	35 ± 7	77 ± 6	81 ± 1	80 ± 9	76 ± 2	
1.0 mM PA	106 ± 6	89 ± 8		90 ± 9	47 ± 1	
1 mM PA +						
0.45 mM SDS	105 ± 20	63 ± 2		83 ± 5	56 ± 6	
0.34 mM SDS	103 ± 10	102 ± 3	72 ± 11	30 ± 6	4 ± 1	
0.22 mM SDS	101 ± 10	90 ± 9	21 ± 4	10 ± 5	6 ± 1	

feasible that both the nature of the packing and the instability of the cationic gold in the media contribute to its irreversible retention into the column. Different ligands (e.g., PA, thiosulfate) have been used for stabilization of cationic forms of elements subjected to separation by micellar electrokinetic [22] or reversed-phase chromatography [23]. The addition of L-cysteine, methionine, and PA to a mobile phase consisting of 0.45 mM SDS at pH 7.5 was studied in order to stabilize ionic gold without affecting the elution of the nanoparticles. PA was finally selected, since using L-cysteine or methionine ionic gold was not eluted or irreproducible results were obtained.

Three concentrations of PA (0.05, 0.2, and 0.5 mM) were investigated. Recoveries obtained for the AuNPs of 10, 50, and 100 nm and ionic gold at different PA concentrations are shown in Table 3. Recoveries in the range of 88–116% were obtained for ionic Au and 10-nm AuNPs at any PA concentration. For 50-nm AuNPs, the recovery was around 100% with a PA concentration of 0.05 mM and decreased down to 76% at the highest PA concentration of 0.5 mM. Recoveries for the 100-nm AuNPs were low at any PA concentration (72–61%), but this was also the case for the manufacturer mobile phase and 0.45 mM SDS mobile phase without PA (69% and 44%, respectively), as it is also shown in Table 3). Best recoveries for ionic Au and AuNPs were obtained by using a PA concentration of 0.05 mM, being used in all further experiments.

Figure 1c shows the chromatogram obtained using a mobile phase containing 0.45 mM SDS and 0.05 mM PA. Using this mobile phase at 1.6 mL min^{-1} , an acceptable resolution of 0.70 between 10-nm AuNP and ionic Au was obtained (see

Fig. 1c), which could be improved by working at lower flow rates (up to 1.03 at 1.0 mL min^{-1} , as it can be seen in Fig. S1 of Supplementary Information (ESM)).

Separation conditions for ionic silver and silver nanoparticles were based on the previous experience gained with gold. To evaluate the behavior of AgNPs in the HDC column, both the manufacturer mobile phase and 0.45 mM SDS were studied by using UV-visible detection. The best recoveries were obtained with the manufacturer mobile phase, ranging from 100 to 92% for 10–60-nm AgNPs (Table S1 of ESM). Therefore, the manufacturer mobile phase with different PA concentrations (0.05, 0.2, 0.5, and 1 mM) was considered for stabilizing ionic silver and studying its separation from AgNPs using ICP-MS detection. Recoveries for the different PA concentrations are shown in Table 3. As it can be observed, good recoveries for ionic Ag (106%) were achieved by using 1 mM PA, higher than for ionic gold (0.05 mM). The recoveries for AgNPs were around 80% for 10 and 40 nm and 47% for 60 nm. In spite of the good recovery obtained by stabilizing the ionic silver with 1 mM of PA, resolution between 10-nm AgNPs and ionic Ag was too low ($R = 0.16$) (Fig. S2 of ESM). The use of a lower flow rate (1 mL min^{-1}) did not improve the resolution in this case ($R = 0.17$). As an alternative for improving resolution, the reduction of the retention time of the nanoparticles by decreasing the ionic strength of the mobile phase was investigated, because at high ionic strength, strong van der Waals interactions dominate over the hydrodynamic effect, increasing the retention times of nanoparticles [19]. Mobile phases with lower ionic strength than manufacturer's one, containing 1 mM PA for stabilization of ionic silver and different SDS

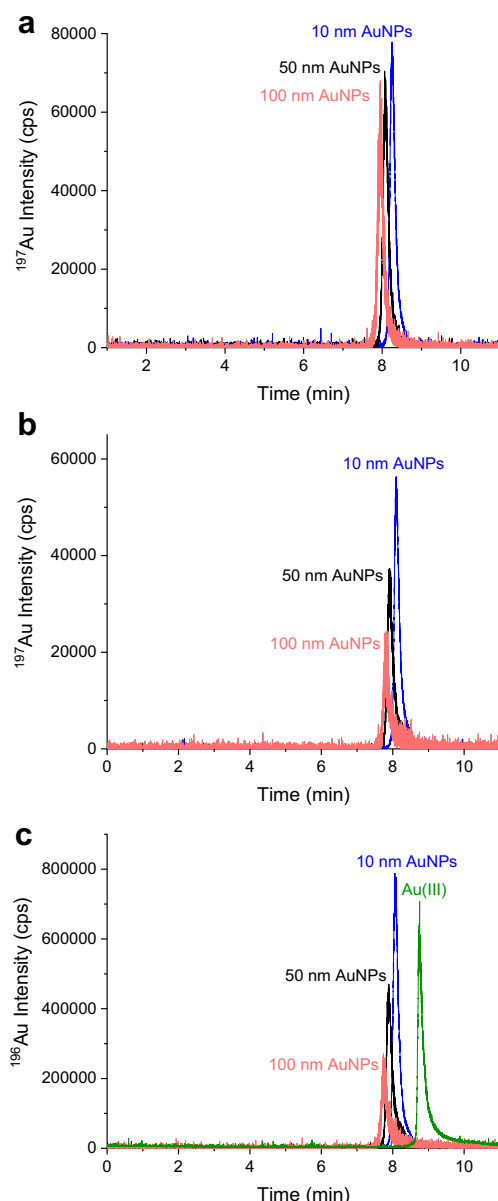


Fig. 1 HDC-ICP-MS chromatograms corresponding to ionic gold and 10-nm, 50-nm, and 100-nm AuNPs ($250 \mu\text{g L}^{-1}$) using different mobile phases. (a) Column manufacturer mobile phase, (b) 0.45 mM SDS, and (c) 0.45 mM SDS and 0.05 mM penicillamine. Flow rate, 1.6 mL min^{-1}

concentrations, were studied. The resolution between 10-nm AgNPs and ionic Ag peaks increased from 0.31 up to 0.93 by decreasing SDS concentrations from 0.45 down to 0.22 mM. Although the best resolution was achieved for the mobile phase containing 0.22 mM SDS and 1 mM PA, recoveries were acceptable just for 10-nm AgNPs and ionic Ag (101 and 90%, respectively), whereas for 20-, 40-, and 60-nm AgNPs, recoveries were below 20%, most probably because of the instability and sorption of the larger NPs onto the column packing material. Thus, a mobile phase containing

0.34 mM SDS and 1 mM PA was selected as a compromise between resolution and recovery. The chromatograms obtained under such conditions are presented in Fig. 2. The resolution achieved between the 10-nm AgNPs and ionic Ag peaks was 0.63, with recoveries for Ag(I), 10-nm AgNP, and 20-nm AgNP of 103%, 102%, and 72%, respectively.

Analytical performance

Both logarithmic [9, 10, 13] and linear [11] fittings (by plotting retention time vs. diameter, or retention time vs. square root of diameter, respectively) have been applied for size calibration in HDC. Although some improvement in linearity has been reported when using the latter approach, no significant differences were observed in this work, with good logarithmic and linear fits, showing correlation coefficient in the range of 0.986 to 1 in both cases (Table S2 of ESM).

The retention times obtained for the different mobile phases studied are summarized in Tables S3 and S4 of the ESM for gold and silver species, respectively. In all cases, retention time repeatability was about 0.1% ($n=3$). Gold and silver mass concentration calibrations were performed by using ionic standards prepared in the mobile phase selected for each element and injected in flow injection mode without column. Flow injection and chromatographic peaks were processed through their peak areas for mass concentration determination, showing good linearity in the range of 50 to $300 \mu\text{g L}^{-1}$ ($R=0.999$).

Limits of detection (LOD) and quantification (LOQ) were related to the detectability of the peaks according to their height, and they were calculated as 3 and 10 times the baseline standard deviation divided by the slope of the peak height calibration. Under such conditions, best-case limits of detection and quantification for gold were 0.05 and $0.16 \mu\text{g L}^{-1}$, respectively, and 0.75 and $2.5 \mu\text{g L}^{-1}$ for silver.

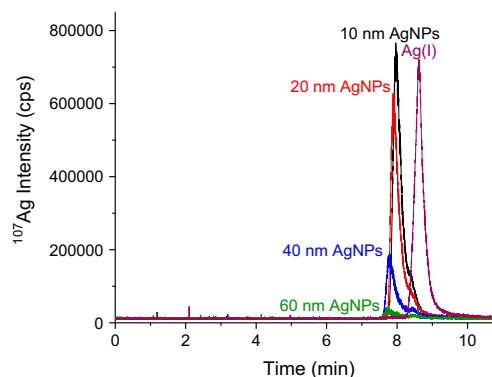
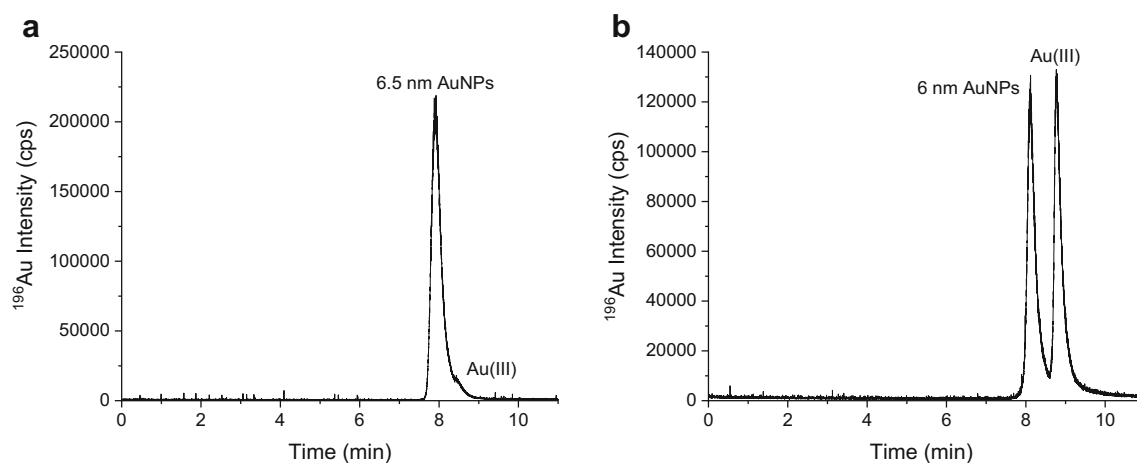
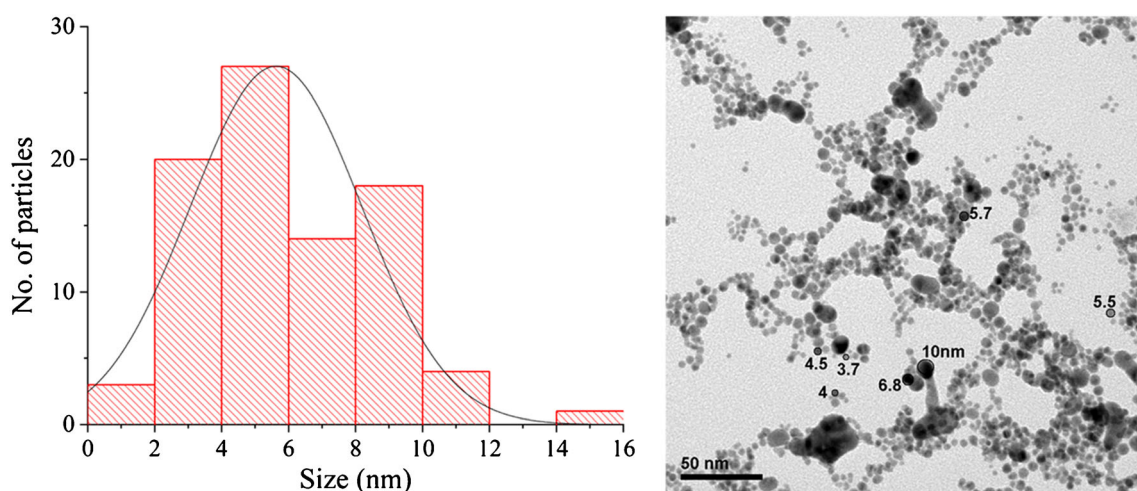


Fig. 2 HDC-ICP-MS chromatograms corresponding to ionic silver and 10-nm, 20-nm, 40-nm, and 60-nm AgNPs using mobile phase containing 0.34 mM SDS and 1 mM penicillamine. Concentration, $250 \mu\text{g L}^{-1}$; flow rate, 1.6 mL min^{-1}

Table 4 Concentration and size of different gold and silver species in nutritional supplements. Mean $\pm$ standard deviation ($n = 3$)

Concentration	HDC-ICP-MS			UF+ICP-MS	ICP-MS	TEM
	AuNPs (mg L ⁻¹)	Au(III) (mg L ⁻¹)	Total Au (mg L ⁻¹)	Au(III) (μg L ⁻¹)	Total Au (mg L ⁻¹)	
Gold supplement	20.4±0.8	(*)	20.4	<2.3	21.0±0.8	
Size						AuNPs (nm)
Gold supplement	6.5±0.1					5.1±2.4
Concentration	HDC-ICP-MS			UF+AAS	AAS	FESEM
	AgNPs (mg L ⁻¹)	Ag(I) (mg L ⁻¹)	Total Ag (mg L ⁻¹)	Ag (I) (mg L ⁻¹)	Total Ag (mg L ⁻¹)	
Silver supplement 1	4.3±1.2	14.2±0.5	18.5	16.8±0.5	21.3±2.1	
Silver supplement 2	(*)	10.9±0.6	10.9	11.6±1.0	12.0±1.6	
Silver Supplement 3	26.3±0.3	(*)	-	1.7±0.6	37.5±0.8	
Size						AgNPs (nm)
Silver supplement 1	23.7±0.8					23.4±5.8
Silver supplement 2	11.6±2.2					14.2±6.7
Silver supplement 3	49.7±0.03					-

(*)Not quantified due to peak overlapping

**Fig. 3** HDC-ICP-MS chromatograms of (a) gold nutritional supplement and (b) spiked with $155 \mu\text{g L}^{-1}$ of ionic gold. Mobile phase, 0.45 mM SDS and 0.05 mM penicillamine. Flow rate, 1.6 mL min^{-1} **Fig. 4** TEM image and size distribution of the gold nutritional supplement

Analysis of gold and silver species in nutritional supplements

One gold and three silver nutritional supplements were analyzed using the mobile phases optimized in the study and the operating conditions described in the “Experimental” section. Table 4 summarizes the concentrations and sizes obtained for the different species found in the nutritional supplements.

According to the manufacturer, the gold nutritional supplement contained 20 mg L⁻¹ of gold as gold nanoparticles of 3.2 nm, whereas ionic gold was absent. Total content of gold was determined by ICP-MS following the procedure described in the “Experimental” section, obtaining a concentration of 21.0 ± 0.8 mg L⁻¹, in agreement with the value given by the manufacturer. The nutritional supplement was analyzed by HDC-ICP-MS using 0.45 mM SDS and 0.05 mM PA as mobile phase. Figure 3a shows the chromatogram corresponding to a 1:150 dilution of the product in mobile phase. The chromatogram consisted of a peak at 7.99 min and a small shoulder at 8.47 min. The peak at 7.99 min corresponds to nanoparticles of 6.5 ± 0.1 nm (using linear fitting calibration), in agreement with the size of 6 nm found by France et al. using MECK-ICP-MS [22]. The small shoulder at 8.47 min would correspond to ionic gold, whose presence was also reported by France et al. [22]. This peak was not resolved from the nanoparticle peak due to the low size of the nanoparticles in the sample and the relative low abundance of the ionic gold. The nutritional supplements were also studied by electron microscopy in order to compare NP sizes. The NP size was determined by calculating the equivalent circle diameter by randomly measuring 87 particles in the images. In this way, the study of the gold supplement by TEM also showed a distribution maximum in a similar size (Fig. 4), with 5.1 ± 2.4-nm diameter, even though some bigger particles appeared in the TEM images. Quantification of the peak corresponding to AuNPs was 20.4 ± 0.8 mg L⁻¹ in the original supplement, in agreement with the total concentration found by ICP-MS (21.0 ± 0.8 mg L⁻¹). Although the determination of Au(III) by ultrafiltration and ICP-MS was attempted, it was below the detection limit of the method (2.3 µg L⁻¹). To check the capability of the method for detection and quantification of ionic gold, the nutritional supplement was spiked with ionic gold (155 µg L⁻¹) (see Fig. 3b). Ionic gold was eluted in the sample at 8.78 min with a mass recovery of 88.7 ± 4.5%.

Three different nutritional supplements containing colloidal and/or ionic silver were analyzed. According to the manufacturer, supplement 1 contained colloidal and ionic silver with a silver concentration of 25 mg L⁻¹, supplement 2 contained colloidal silver with a concentration of 10 mg L⁻¹ and a size range between 0.6 and 5 nm, and

supplement 3 contained colloidal silver with a concentration of 30 mg L⁻¹ and a size range between 1 and 100 nm. The three supplements were firstly analyzed by ultrafiltration and flame atomic absorption spectroscopy (FAAS) to determine ionic and total silver, obtaining the concentrations shown in Table 4. The three nutritional supplements were analyzed by HDC-ICP-MS using the mobile phase containing 0.34 mM SDS and 1 mM PA at a flow rate of 1.6 mL min⁻¹ after dilution in the same mobile phase. The chromatograms obtained are shown in Fig. 5. The chromatogram of supplement 1 showed two peaks at 7.99 and 8.48 min, corresponding to AgNPs of 23.7 ± 0.8 nm and ionic Ag. In this case, with AgNPs of 23.7 nm, good

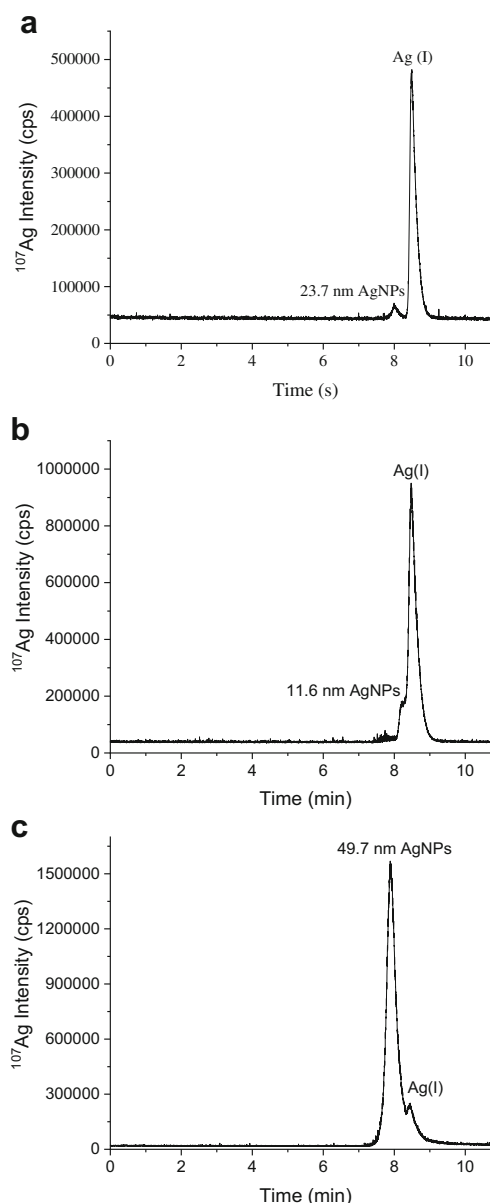


Fig. 5 HDC-ICP-MS chromatograms of silver nutritional supplements. (a) supplement 1, (b) supplement 2, and (c) supplement 3. Mobile phase, 0.34 mM SDS and 1 mM penicillamine. Flow rate, 1.6 mL min⁻¹ flow

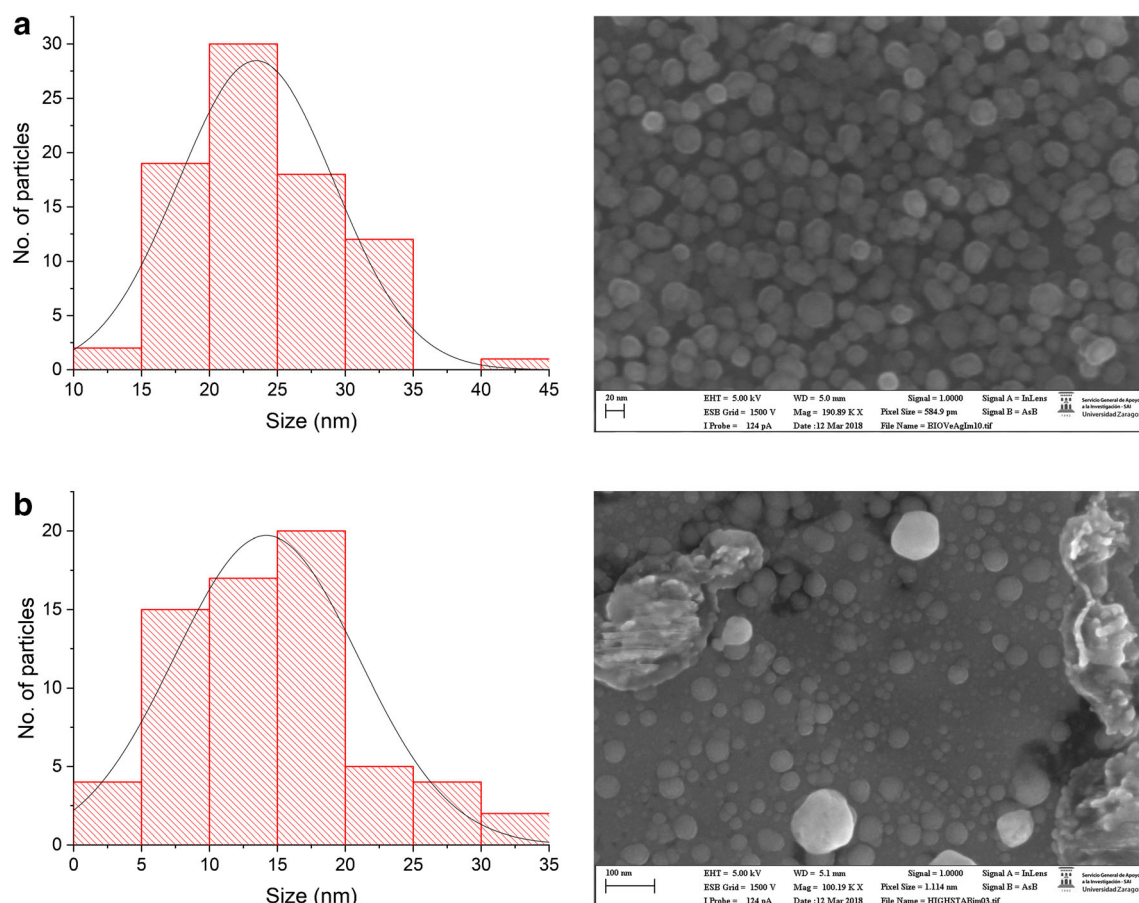


Fig. 6 FESEM images and size distributions of silver nutritional supplements 1 (**a**) and 2 (**b**)

resolution was obtained between AgNPs and ionic Ag, and the quantification gave good results as it is discussed below. The chromatogram of supplement 2 just showed a peak at 8.46 min with a small shoulder at 8.1 min which can be assigned to ionic Ag and AgNPs of 11.6 ± 2.2 nm, whereas the chromatogram of supplement 3 showed two peaks at 7.89 and 8.48 min, corresponding to AgNPs of 47.9 ± 0.03 nm and ionic Ag, respectively. In all cases, size linear fitting calibrations have been used.

The silver supplements were studied by FESEM, due to the expected bigger AgNP sizes. Figure 6 shows FESEM micrographs from supplements 1 and 2. AgNP size distributions were also calculated using ImageJ software (diameters of 82 and 67 particles were randomly measured, respectively, for supplements 1 and 2), showing mean sizes of 23.4 ± 5.8 and 14.2 ± 6.8 nm, which were in fair agreement with the sizes obtained by HDC-ICP-MS (see Table 4). AgNPs in supplement 1 were quite regular in size. However, in FESEM images, silver distribution of supplement 2 was heterogeneous. No reliable electron images were obtained for Supplement 3, most probably due to the composition of the sample.

The concentrations of different silver species obtained for each supplement are shown in Table 4, where results obtained for total and ionic silver by ultrafiltration and

FAAS are also included for comparison. HDC-ICP-MS quantification for supplements 1 and 2 was in good agreement with the results obtained by ultrafiltration and FAAS. The small shoulder obtained for supplement 2 by HDC-ICP-MS, corresponding to 11.6-nm AgNPs, was not quantified due to the overlapping with the dissolved silver peak. Different batches from the same supplements had been previously analyzed by anodic stripping voltammetry [24], obtaining similar results. The presence of dissolved silver together with larger amounts of AgNPs was confirmed in supplement 3, although dissolved silver could not be quantified properly due to the overlapping with the peak corresponding to the nanoparticles. In this supplement, concentrations obtained by HDC-ICP-MS were lower than the total silver concentration determined by FAAS due to the low recoveries achieved for nanoparticles over 40 nm.

Conclusions

Although HDC-ICP-MS has been used successfully for the separation and quantification of inorganic nanoparticles, this hyphenated technique suffers from the inherent low resolution of hydrodynamic chromatography. However, the capability of

HDC-ICP-MS for the simultaneous determination of dissolved species and nanoparticles of the same element had still not been exploited in depth. In this study, a HDC-ICP-MS methodology has been developed for the separation and determination of dissolved species and nanoparticles of gold and silver, by stabilizing the ionic forms of both elements by adding penicillamine to the mobile phases. Using a mobile phase containing penicillamine at 0.05 mM, ionic gold was eluted quantitatively from the HDC column, whereas a higher concentration, up to 1 mM, was required for ionic silver. Fair resolution (ca. 0.7–0.6) between dissolved species and 10-nm metallic nanoparticles was obtained with the optimized mobile phases, although the partial overlapping of the peaks corresponding to dissolved species and nanoparticles below 10 nm can hamper the quantification of the lower abundance species, especially when the latter are present at concentrations closed to the limit of quantification. In such cases, decreasing of the elution flow rate can improve the resolution but at the cost of increasing the separation time.

Using the developed methods, it was possible to detect the presence of nanoparticulate and/or dissolved species of gold and silver at the microgram per liter level in less than 10 min, as well as the simultaneous determination of the nanoparticle size and the mass concentration of both species, although the latter depending on their relative abundance and the size of the nanoparticles present. The analysis of different dietary supplements containing gold and silver confirmed the presence of nanoparticulate and/or dissolved species in the samples. The sizes of the gold and silver NPs found by HDC-ICP-MS in the dietary supplements were in good agreement with those found by TEM or FESEM, respectively. Quantitative results were compared with those obtained by ultrafiltration and AAS, although the quality of the results obtained depended on the relative abundance of the species in the sample and the size of the nanoparticles if present. Despite their limitations, the HDC-ICP-MS methods developed can be considered a feasible alternative to other methods based on techniques like single-particle ICP-MS, where the detectability of nanoparticles is more seriously affected by the presence of dissolved species.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00216-020-03132-3>.

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Authors' contributions M.S. Jimenez is the supervisor and coordinator of all experimental work and data revision. She did the experimental work regarding the analysis of gold dietary supplement. She is responsible of writing the manuscript.

M. Bakir is responsible for the experimental work regarding the characterization and quantification of silver nanoparticles and dissolved silver

and the analysis of silver dietary supplements. She is the author of all figures.

D. Isabal did the experimental work regarding the characterization of gold nanoparticles and dissolved gold.

M.T. Gómez is the coordinator of the work in the group laboratory, being responsible for the supplies and has collaborated in the writing and revision of the manuscript.

J. Perez-Arantegui is responsible for FESEM and TEM measurements and data interpretation. She has collaborated in the revision of the manuscript.

F. Laborda is the main researcher of project RTI2018-096111-B-I00 (MICINN/FEDER). He has supervised the experimental work and the writing and revision of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Code availability Not applicable

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CRITICAL REVIEW

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Analytical applications of single particle inductively coupled plasma mass spectrometry: a comprehensive and critical review

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Single particle inductively coupled plasma mass spectrometry (SP-ICP-MS) refers to the use of ICP-MS as a particle counting technique. When ICP-MS measurements are performed at very high data acquisition frequencies, information about (nano)particles containing specific elements and their dissolved forms can be obtained (element mass per particle, size and number and mass concentrations). As a result of its outstanding performance, SP-ICP-MS has become a relevant technique for the analysis of complex samples containing inorganic nanoparticles. This review discusses the maturity level achieved by the technique through the methods developed for the detection, characterisation and quantification of engineered and natural (nano)particles. The application of these methods in different analytical scenarios is comprehensively reviewed and critically discussed, with special attention to their current technical and metrological limitations. The emergent applications of SP-ICP-MS in the field of nanoparticle-tagged immunoassay and hybridization methods are also reviewed.

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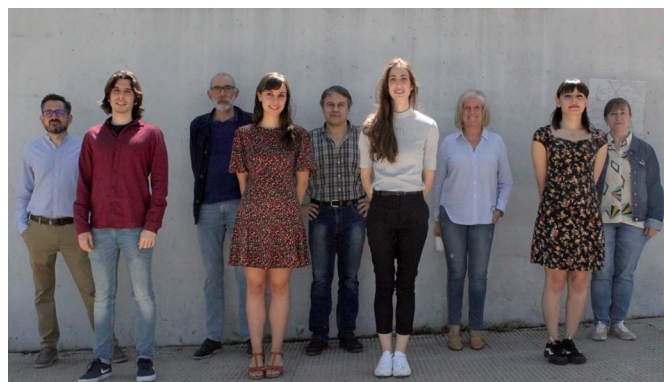
SP-ICP-MS: a fast-developing technique

The use of inductively coupled plasma mass spectrometers in single particle mode has led to the emergence of a particle counting technique known as single particle inductively coupled plasma mass spectrometry (SP-ICP-MS). Although the origins of the technique can be traced back to the 1970's,¹

a series of key papers by Degueldre *et al.*,^{2–6} published between 2003 and 2006, are considered its starting point. However, the application of SP-ICP-MS to the analysis of engineered nanomaterials,^{7,8} followed by its implementation in commercial ICP-MS instruments,^{9–11} became the main driving force behind the success of the technique in recent years.

The birth and evolution of SP-ICP-MS resembles that of the ICP-MS technique itself (Fig. 1). After their initial conception, it took some time to get the first ICP-MS research instrument, as well as the first application to nanomaterials in the case of SP-ICP-MS. But from then on, it took only about three years for manufacturers to launch the first commercial ICP-MS instruments, or the first

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The authors belong to the Group of Analytical Spectroscopy and Sensors (GEAS) of the University of Zaragoza. The main research area of the group lies in the development and application of analytical techniques and methods in Nanometrology for the detection, characterization and quantification of natural and engineered nanoparticles. The group has made significant contributions in single particle ICP-MS and flow field flow fractionation hyphenated to ICP-MS. The authors have a long experience in these techniques but also in hydrodynamic chromatography, electron microscopy, as well as X-ray and electroanalytical techniques, which leads to a broad analytical platform for solving complex problems involving nanoparticles and colloids. The group has more than 10 year of experience and 40 publications in this field of Analytical Nanometrology.

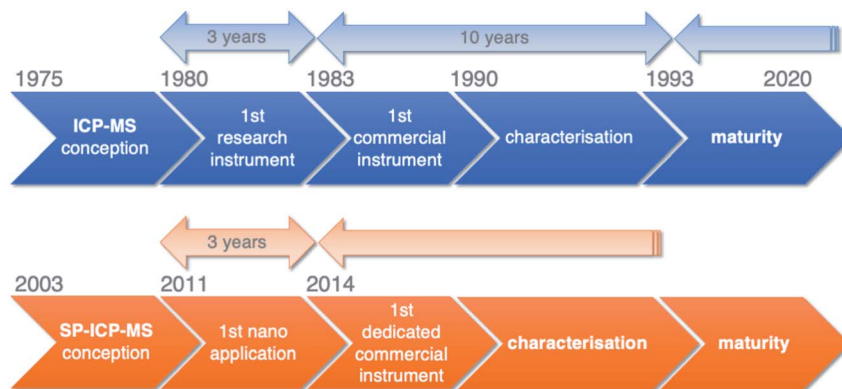


Fig. 1 Comparison of the evolution of SP-ICP-MS with respect to ICP-MS (adapted from Horlick¹²).

dedicated instruments in the case of SP-ICP-MS. Thereafter, conventional ICP-MS evolved in a fast way, becoming a mature technique in about ten years.¹² According to Horlick, a mature technique is characterised by the fact that their methods are applied to a wide range of analytical problems and determinations, and become established as a routine tool with broad commercialisation. Before the maturity stage, a technique goes through a characterisation stage, a period of intense research activity aimed at achieving a complete picture of the technique at instrumental, metrological and methodological levels. On the other hand, during the characterisation stage the new technique seems to have no limits and to be able to solve all the problems, whereas with maturity comes the realization that not all is ideal and there are a number of problems that will not be solved. In the case of SP-ICP-MS, the question is whether the technique can be considered to have reached maturity or is still in the characterisation phase. The final aim of this comprehensive review is to gather the publications involving SP-ICP-MS and critically evaluate the current state of the technique, its limitations and its level of maturity level. Special attention is paid to the problem-solving analytical methods developed and their application under different relevant analytical scenarios.

Basic principles

When an ICP-MS instrument is used at very high reading frequencies, ICP-MS becomes a particle counting technique, being able to deliver information in a particle-by-particle basis. The basics of SP-ICP-MS measurements were established by Degueldre *et al.*² Basically, when a diluted suspension of nanoparticles is nebulized into an ICP-MS, and an isotope of the element present in the nanoparticles is measured at acquisition frequencies over *ca.* 100 Hz, a series of events corresponding to individual nanoparticles are recorded over a continuous baseline. The intensity of these events is related to the mass of the element in the nanoparticle, and hence to its size if additional information about the composition, shape and density of the nanoparticle are available, whereas the frequency of events is proportional to the number concentration of nanoparticles. Subsequently, Laborda *et al.*⁷ revealed that information about dissolved forms of the element measured could also be obtained from the baseline. Whereas the dissolved species are homogeneously distributed in all the aerosol droplets, the

nanoparticles are present randomly in some of them. Thus, a constant signal (baseline) is produced by the dissolved species, whereas the nanoparticles give rise to individual signal events, as it can be seen in a typical time scan (Fig. 2a).

As the SP-ICP-MS measurements are performed in time resolved mode, the data acquisition frequency (or the number of events along a fixed acquisition time), controlled through the dwell time of the instrument, is one of the most relevant parameters. Considering that the ion cloud generated in the plasma from a single nanoparticle can be detected during 300–1000 μs ,^{13,14} nanoparticle events can be recorded in two different forms, depending on the dwell time selected. When using dwell times in the millisecond range (3–10 ms), larger than the duration of the ion cloud in the instrument, events are recorded as one-reading signals (pulses), whereas for dwell times in the microsecond range (10–200 μs), they are recorded as peaks (transient signals), comprising several readings (see the insets in Fig. 2a). For pulses, the intensity event is given by the reading itself, whereas for peaks, the intensity is calculated as the sum of the individual readings along it. With respect to the acquisition frequency, it is worth to mention that the commercial launch of SP-ICP-MS in 2014 was linked to quadrupole spectrometers with higher reading frequencies, allowing dwell times down to 10 μs ,¹⁰ instead of just working at milliseconds. In the meantime, time-of-flight⁹ and double focussing¹¹ spectrometers have become commercially available with minimum dwell times of 33 and 10 μs , respectively.

Raw data from both milli- and microsecond time scans can be processed by plotting the event intensity *vs.* the number of events, obtaining histograms as shown in Fig. 2b, where the first distribution is due to the background and/or the presence of dissolved forms of the element measured and the second to the nanoparticles themselves. The second distribution is further processed to convert the event intensities to mass of element per nanoparticle or size distributions (Fig. 2c). Instrumental and metrological issues related to measurements, data acquisition and data processing have been summarized and described in detail in a number of reviews^{1,15–18} and articles therein. It is worth to mention that the different types of quantitative information on nanoparticles achievable by SP-ICP-MS, and discussed in the next section, require different calibrations with nanoparticle size and number concentration

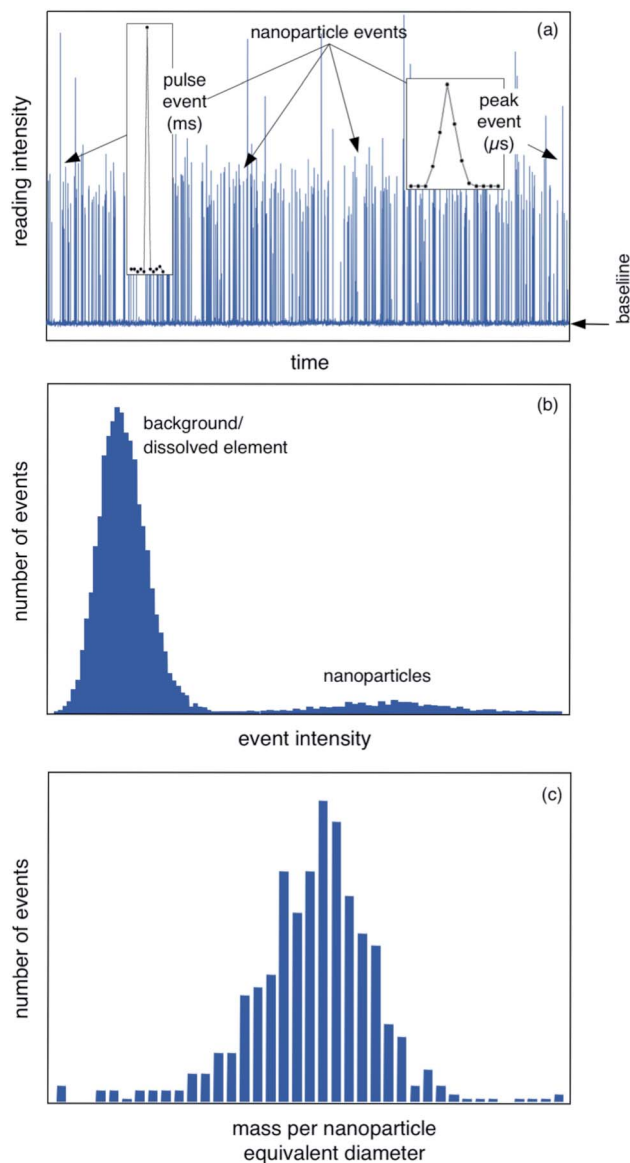


Fig. 2 (a) Time scan of suspension containing nanoparticles and dissolved forms of the same element. (b) Event intensity histogram of data from (a). (c) Mass per nanoparticle/size distribution of spherical nanoparticles calculated from the second intensity distribution in (b).

standards. If size standards of the particles under study are not available, indirect calibrations based on the use of dissolved standards are usually applied. In these cases, sample introduction flow rate and the analyte nebulization efficiency have to be known to determine the mass of element per nanoparticle and hence the nanoparticle size. The analyte nebulization efficiency is commonly determined using the methods developed by Pace *et al.*⁸ An overview of calibration issues in SP-ICP-MS can be found in Laborda *et al.*¹⁹

Analytical information: analytes and measurands

The strength of SP-ICP-MS lies in the different types of analytical information that the technique can provide from very

simple measurements. As we have seen above, two types of analytes are under the scope of SP-ICP-MS, namely (nano) particles and dissolved species. The term “nanoparticle” is going to be used here in a broad sense, although in most cases it will coincide with the ISO definition (nano-object with its three dimensions in the nanoscale, 1–100 nm).²⁰ Other nano-objects, like nanofibers/nanotubes or nanoplates (two and one dimensions in the nanoscale, respectively) may be suitable of being analysed by SP-ICP-MS, depending on the magnitude of the other dimensions that are not in the nanoscale. Thus, particles over 100 nm, even in the micrometre range, have been successfully analysed, although issues related to their nebulization and ionization must be considered.²¹

In any case, the information provided by SP-ICP-MS can be: (i) qualitative, about the presence of (nano)particulate and dissolved forms of specific elements; (ii) quantitative contents, as number and mass concentrations; and (iii) characterisation, about the mass of element/s per nanoparticle and nanoparticle size.

For dissolved species, the mass concentration of the element monitored is the measurand of interest, whereas for nanoparticles, the primary measurands of concern are the number concentration and the mass of element per particle. When additional information about the shape, composition and density of the particles is available, information about the size of the particles can be obtained. Nevertheless, a spherical shape is commonly assumed and the equivalent spherical diameter is the measurand usually reported. Any population of particles always exhibits more or less broad size distributions, recorded primarily by SP-ICP-MS as element content distributions, whose measurands are histograms showing the number of particles for each of a number of defined size or element content per particle classes, respectively. Table 1 summarises the quantitative and characterisation information with the corresponding measurands for each of the analytes involved in SP-ICP-MS. Qualitative information about the presence/absence of dissolved species and/or nanoparticles over a certain concentration and size cannot be disregarded because of the practical limitations of the technique when analysing complex samples, as it will be shown below. In addition, other types of information can be obtained from SP-ICP-MS measurements (*e.g.*, porosity,²² density,²³ aggregation,^{24,25} agglomeration²⁶), although they will not be discussed in detail.

Scientific production and evolution stage

Since the publication of the first article by Degueldre and Favarger in 2003, almost 400 studies directly related to SP-ICP-MS have been published in peer-reviewed journals until the end of 2020. These publications include a limited number of reviews, tutorials and divulgation articles; whereas most of them consists of articles on fundamentals and basic aspects of the technique, the development of methods, as well as their application. As it has been stated above, the analysis of engineered nanomaterials is behind the success of SP-ICP-MS, and

Table 1 Analytes, analytical information and measurands involved in SP-ICP-MS

Analytical information		Analyte	Measurand
Qualitative	Presence/absence	Nanoparticle	
		Dissolved element	
Quantitative content	Concentration	Nanoparticle	Number concentration
	Concentration	Dissolved element	Mass concentration of nanoparticulate element Mass concentration of dissolved element
Characterisation	Element content	Nanoparticle	Element mass per particle
	Size	Nanoparticle	Equivalent spherical diameter
	Element content distribution	Nanoparticle	Histogram showing the number of particles for each of a number of defined element content per particle classes
	Size distribution	Nanoparticle	Histogram showing the number of particles for each of a number of defined size classes

most of the works deal with manufactured nanoparticles, although natural and incidental nanoparticles have also been analysed. SP-ICP-MS has proved its usefulness in other areas, like nanoparticle tag-based immunoassay and hybridization methods, as it will be discussed in a specific section.

The scientific production from 2003 to the end of 2020 has been organized in four fields, namely “Reviews”, “Fundamentals”, “Method Development” and “Applications”. With respect to the “Reviews” field, one tutorial,¹⁵ five reviews^{1,16–18,27} and two divulgation articles^{28,29} on the specific topic of SP-ICP-MS have been published to date. However, the technique is being included in most reviews on ICP-MS^{30,31} or about the analysis of natural³² or engineered^{33–35} nanomaterials. The “Fundamentals” field covers publications whose main objective is the study of basic aspects of the technique (*e.g.*, instrumentation, plasma processes, sample introduction, data acquisition and processing). All these studies have been performed with nanoparticle standards, although synthetic matrices have been considered in some cases, but always under controlled laboratory conditions. The “Method Development” field includes publications focused on the development of proofs-of-concept methods, based on the use of in-lab synthesized nanoparticles, nanoparticle standards or samples spiked with standards. When the method has been applied to samples containing original nanoparticles, it has been considered under the “Applications” field. Analysis of samples from laboratory tests (*e.g.*, *in vivo*, *in vitro*, migration) involving nanoparticle standards have also been considered within this field, as it will be justified below.

Fig. 3 summarizes the distribution of publications in the four fields cited above directly related with the analysis of nanomaterials or samples containing nanomaterials, as well as their chronological evolution. The analysis of these data may give light about the maturity and the expected evolution of SP-ICP-MS. As it has been stated above, the rise of SP-ICP-MS publications started from 2011, with a constant increase in the total number of publications related to both “Fundamentals” and “Method Development” until 2016, showing some stabilization in recent years, whereas the number of publications about “Applications” is steadily growing. Following the

evolution stages proposed by Horlick, and despite some overlap between them is unavoidable, the trend in publications suggests that SP-ICP-MS has passed the stage of characterisation and would be at the beginning of the maturity stage. However, other indicators than the extent of application of the technique, namely metrological indicators like method validation and traceability, should also be considered. While traceability of results is out of question in conventional ICP-MS because of the availability of validated methods, standards and reference materials,³⁶ this is not the case in SP-ICP-MS. As will be seen in the following sections, the number of SP-ICP-MS methods developed for the most diverse applications is increasing, although the number of validated ones is still limited. On the other hand, due to the special features of nanoparticles, the availability of standards and reference materials is still scarce, conditioning in the end the traceability of the results obtained.

Analytical methods based on SP-ICP-MS: validation

In spite of the significant evolution of the technique discussed above, method validation in SP-ICP-MS is still at an early stage,

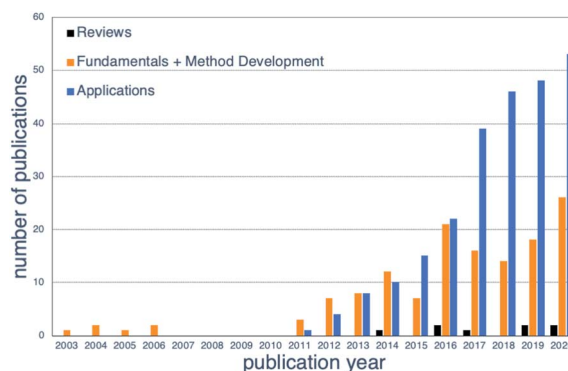


Fig. 3 Evolution of SP-ICP-MS publications related to reviews, fundamentals, method development and applications.

lacking the harmonization of other fields in analytical chemistry, mainly because of the special features of nanoparticles as analytes. Whereas conventional analytes consist of identical entities (mostly atoms in ICP-MS) identified by their chemical composition (e.g., silver, titanium dioxide), identification of particles (e.g., 10 nm spherical silver or titanium dioxide nanoparticles) requires determining their chemical composition but also their morphology (size and shape). This means that validation parameters, like limits of detection and quantification, working range, precision or trueness, must be applied to mass/number concentrations but also to particle size. With respect to size, it must keep in mind that particles always exhibit more or less broad size distributions, entailing that particles cannot be considered as truly identical entities. An additional drawback in relation to validation is the lack of certified reference materials for nanoparticles in any type of matrix, which forces to use recovery tests with matrix-free reference materials or properly characterised commercial nanoparticles for the evaluation of trueness. These recovery tests, based on the spike of nanoparticles, must test not only the recovery of the mass added, but also that the particle size and its distribution remain unchanged. For all these reasons, Linsinger *et al.*³⁷ proposed an adaptation of conventional validation schemes to the detection and quantification of engineered nanoparticles, although the number of validated methods already published is still scarce. In most cases, the scheme has been based on the addition of nanoparticles to different matrices, such as surface waters,³⁸ fruit juices,³⁹ chicken meat,⁴⁰ human liver and spleen tissues⁴¹ or human blood.⁴² In these cases, particle stability in the matrices has a strong influence on trueness and precision of the results, as it was reported for silver nanoparticles in chicken meat, since metallic nanoparticles were transformed into silver sulfide.⁴⁰ Only in a few cases, samples with nanoparticles originally present, such as Ag nanoparticles in confectionery,⁴³ have been described in validated methods. Finally, some methods for screening purposes have also been validated for detection of silver and gold nanoparticles in foods⁴⁴ and TiO₂ nanoparticles in confectionery products.⁴⁵

Validation parameters commonly determined in these studies include trueness, selectivity, precision, detection limits, linearity and robustness.³⁷ The evaluation of trueness has been done mostly by spiking control samples with nanoparticle standards, ensuring that the particle size and size distribution remain unchanged, together with the recovery of the mass added. In general, larger bias, as high as +80% have been reported for number concentration compared to particle size, with bias around -20%.¹⁹ Comparison with nominal median diameter and/or concentration of the standard added is the common rule, although the confirmation of the results by independent methods is highly recommended when possible. Thus, validation of the size characterisation has been performed by comparison with electron microscopy techniques (TEM or SEM), such in the characterisation of a series of TiO₂ materials used as food additives⁴⁶ or with the gold nanoparticle reference material RM8012,⁴⁷ showing differences on size distributions.

Selectivity has been evaluated considering possible (poly) atomic interferences^{38,40,41} and other matrix constituents, which may cause changes in nebulization efficiency or in the ionization, affecting to particle concentration and size, respectively.⁴⁸ In any case, the effect of other nanoparticles or nanoparticles of the same composition but with different properties (size, coating...) should also be evaluated.

Respect to precision, determination of the nanoparticle number concentration is usually less precise than size determination, with reproducibility that reach up to 90% in some cases.⁴⁹ Data processing, is the driving factor in this parameter over sample preparation or sample dilution, especially at high baseline levels that make discrimination of nanoparticle readings more difficult.⁴³ It should be noted that satisfactory results have been obtained when comparing the repeatability and reproducibility obtained with the values predicted by the Horwitz ratio in the analysis of TiO₂ particles at concentration levels in the order of 0.01 mg Ti per kg.⁴¹

Despite there are not expressions widely accepted for the calculation of the limits of detection in the different domains covered by SP-ICP-MS, Laborda *et al.*¹⁷ have proposed a harmonizing approach to calculated LODs for validation purposes. Most widely applied criteria for calculation of size detection limits are based on the use of multiples of the baseline standard deviation (*n*-sigma criterion). Multiples from 3- to 8-sigma criteria can be found in SP-ICP-MS publications,⁴¹ although the use of 5-sigma criteria has been justified by these authors¹⁷ on a routine basis. In general, size LODs depend on different factors, such as the concentration of the dissolved element, the strategy used for subtracting the baseline signal to the particle signal or the type of mass spectrometer.⁴⁵ A comprehensive list of size LODs for metallic and oxide nanoparticles using a commercial quadrupole instrument under typical experimental conditions can be found in Laborda *et al.*¹⁷ Size LODs around 10 nm and larger were obtained for most common nanoparticles at 100 μ s dwell times. Incomplete vaporization of large particles in the plasma can limit the linearity of the size measurement ranges, hence larger particles, in the micrometre range, can produce signals outside the linear range of the detector.⁵⁰ For instance, a linear size range up to 150 nm has been reported for gold nanoparticles, whereas for SiO₂ it can increase up to 1000 (ref. 51) or 2000 nm.⁵⁰ Besides, particles larger than 2–5 μ m are transported into the plasma with lower efficiency.

With respect to the number concentration LOD, it depends on the number of particles events detected in the blank, the sample introduction to the ICP-MS and the acquisition time. The use of conventional procedures for concentration LOD based on the blank standard deviation as in size LOD results in unrealistic values^{9,42} and therefore, they cannot be used interchangeably. LOD values of 100 particles per mL have been reported¹⁷ with acquisition times of 60 s, using a cyclonic spray chamber and a concentric nebulizer. The number concentration measurement range is limited by the occurrence of multiple particle events, which cause a progressively loss of linearity. The occurrence of events due to two or more nanoparticles is predicted by Poisson statistic and is expected to be

significant, depending on the nebulization system and the range of dwell times (milli or microseconds) used.¹³ Linear ranges up to 10^7 L^{-1} can be achieved with conventional nebulization systems at millisecond dwell times, which can be increased one order of magnitude working at microsecond dwell times.

Robustness has been evaluated commonly by changing different parameters that affect the measurement results, such as sample pre-treatment or dilution factor,⁴⁰ although in some cases only the element total concentration has been considered for robustness evaluation.⁴¹

Other studies have included quality control procedures, such as the evaluation of trueness by spiking nanoparticles standards to natural matrix samples, considering that conventional procedures cannot be directly applied in SP-ICP-MS analysis due to the limitations described above. Thus, trueness has been evaluated by measuring recovery of the spiked nanoparticles, mainly based on number concentration, and the use of alternative techniques to confirm size results, such as for the sizing and quantification of Ag nanoparticles in food simulants,⁵² moisturizing creams,⁵³ fish tissue,⁵⁴ or ZnO⁵⁵ and TiO₂ nanoparticles⁵⁶ in natural waters. This strategy has also been applied to the detection of naturally occurring iron oxide nanoparticles in crude oil and residual fuel oil by spiking in-lab synthesized silica-shelled Fe₃O₄ nanoparticles.⁵⁷ Alternatively, the agreement between the total element content determined by acid digestion and ICP-MS can be used for confirmation of the results, as in the determination of Ce, Cd and Pt-bearing nanoparticles in road runoff sediments⁵⁶ or Au nanoparticles in tumour cells after alkaline digestion.⁵⁸

However, if the sample contains nanoparticles smaller than the LOD,^{54–56} size information will be overestimated and number/mass concentration underestimated in consequence. In such situations, SP-ICP-MS should be restricted to confirm the presence of nanoparticles containing the element monitored over a certain equivalent diameter and concentration.

Applications of SP-ICP-MS: analytical scenarios

In relation to the application of SP-ICP-MS to real-world analytical problems, Fig. 4 shows four different analytical scenarios that can be considered. Whereas the first one (type 0) corresponds to the analysis of pristine nanoparticles commonly produced at laboratory or industrial-scale, which just involves their characterisation, the other three correspond mainly to the needs of analytical information arising from the risk assessment of nanomaterials and nanoproducts in relation to environment, health and safety, as well as to legal regulations, which involve the detection, characterisation and/or quantification of nanoparticles in the presence of, more or less, complex matrices. Type 1 scenario includes the analysis of industrial and consumer products containing nanoparticles (*e.g.*, cosmetics, textiles, polymers). Type 2 scenarios consider laboratory tests with pristine nanoparticles or products containing nanoparticles to assess their release, fate, and behaviour (2.1) as well

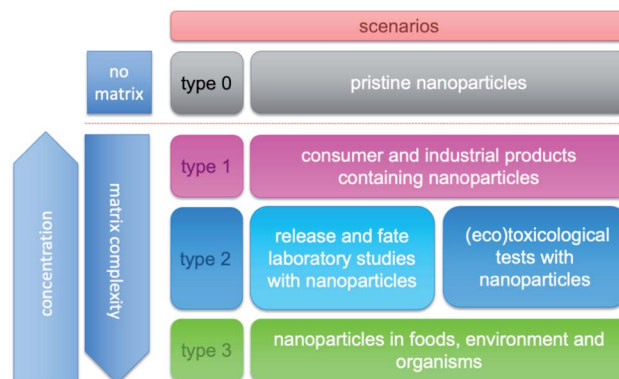


Fig. 4 Analytical scenarios related to nanoparticles (adapted from Laborda *et al.*⁵⁹).

as their (eco)toxicity in a variety of exposure conditions (*e.g.*, environmental micro and mesocosms, migration in food simulants, gastrointestinal digestions, *in vitro* and *in vivo* (eco) toxicity test) (2.2). Finally, type 3 scenarios are related to the monitoring of the occurrence of nanoparticles in foods (3.1) environment (3.2) and organisms (3.3).

Leaving aside the scenario type 0, where the nanoparticles are the sample because of the lack of matrix, each of the three other scenarios involve samples containing nanoparticles and, in general terms, represent a progressive increase in analytical complexity, from the point of view of both the matrix and the decreasing concentrations.

The use and application of SP-ICP-MS methods has been organized according to the scenario classification presented in Fig. 4. Fig. 5 shows the distribution of the number of publications in relation to these different scenarios. The chart shows that two thirds of applications correspond to laboratory tests (scenario 2), whereas one fourth involves samples originally containing nanoparticles (scenario 3). The analysis of pristine nanomaterials (scenario 0) and products containing nanoparticles (scenario 1) account for 16%, most probably because they are usually analysed by other well-established techniques (*e.g.*, electron microscopy, DLS). The following sections provide a detailed reviewing of the SP-ICP-MS application in the different scenarios considered.

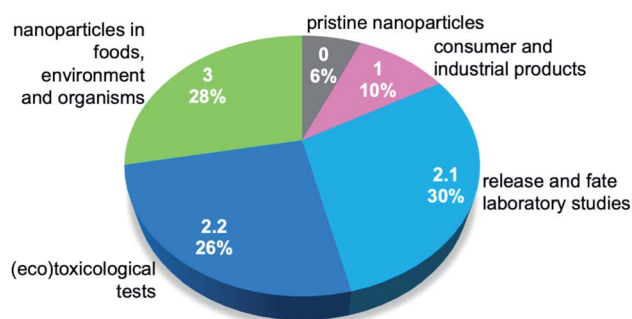


Fig. 5 Distribution of publications (2011–2020) related to applications of SP-ICP-MS in different analytical scenarios.

Scenario 0: analysis of pristine nanoparticles

The analysis of pristine nanoparticles is regarded as the least complex scenario, since there is no matrix in which they are contained. Engineered nanomaterials commonly produced at laboratories for research or industrial purposes,^{61,63–72,75,76} but also commercial suspensions,^{60,62,73,74} have been considered in this section. Table 2 summarizes the applications of SP-ICP-MS to the analysis of such pristine nanoparticles. In any case, the main aim of the analysis is focused on the characterisation of the nanoparticles, although the effect on size of different factors (stability,⁶⁰ kinetics,⁶¹ use of specific reagents^{67,76}) along their synthesis have also been studied by some authors.

Pure nanoparticles of metallic Au,^{63–65} Ag,^{60,61,63} Pd and Ni,⁶⁸ as well as TiO₂,⁷¹ have been analysed by SP-ICP-MS. The nanoparticles were usually obtained by chemical synthesis, although they were also produced by milling.⁶⁰ Most of the nanoparticles were available as suspensions, however, those presented as powders had to be prepared as stable suspensions, requiring convenient dilutions in all cases. Only in the work by Lahtinen *et al.*⁷⁶ nanoparticles had to be extracted with water, because they were obtained by reduction of tetrachloroaurate adsorbed onto 3-D filters.

In relation to the analytical information delivered, size distributions and mean/median sizes were typically reported, although mass^{61,68} and number concentration^{64,68} were also considered when samples were originally presented as suspensions. In this scenario, characterisation was not only limited to sizing. Kálomista *et al.*⁶⁵ were able to provide information about the shape of the nanomaterial, differentiating between spherical nanoparticles and nanorods of Au by comparing the signal-time profiles of the peaks recorded. Merrifield *et al.*⁶² characterised and quantified Au–Ag core–shell nanoparticles, whereas Kéri *et al.*,⁶³ following a similar methodology to Kálomista,⁶⁵ were able to distinguish between Au–Ag alloyed and core–shell nanoparticles, providing average Au : Ag molar ratios.

The analysis by SP-ICP-MS of composite particles consisting of polymer⁶⁶ or silica⁷² particles containing metallic nanoparticles allowed to obtain the mass of Au or Pt per particle, respectively, as well as the corresponding distributions and the number of metallic nanoparticles per composite particle when their size was known. Complex nanomaterials, like up-conversion nanoparticles (NaYF₄ and NaGdF₄ doped with Yb or Er), could also be characterised by decreasing the element mass per particle detection limit; this could be done by reducing the resolution of the quadrupole and hence increasing the transmission of ions.⁷⁰

Although carbon nanotubes are not directly detectable by SP-ICP-MS, their metal impurities have been used as proxies for their detection. In that way, the yttrium contained in nanotubes allowed their detection,^{73,74} although the concentration was underestimated due to the difficulties of detecting nanotubes with low loads of yttrium.

The characterisation of pristine nanoparticle by SP-ICP-MS has been complemented by electron microscopy techniques for size and shape characterisation^{57,61,64,65,68–70,73,74} and

XRD^{68,69,72,75} for confirmation of their nature through their crystalline structure.

Scenario 1: analysis of consumer and industrial products containing nanoparticles

Owing to their specific properties at the nanoscale level, nanoparticles are currently contained in numerous consumer and industrial products. The nano-enhanced products analysed by SP-ICP-MS are summarized in Table 3; they consist of consumer, mainly cosmetics,^{53,77–85} and industrial products, including food additives^{43,46,89} and other materials, like steel,⁹⁰ petroleum products^{57,91} or tattoo inks.^{92,93}

Nanoparticles in cosmetics are mainly focused on sunscreens,^{77–79,84} in which TiO₂ is the most frequent nano-component due to its photocatalytic properties, although ZnO has also been studied in this type of products.^{80,81} TiO₂ was the target analyte in other cosmetics and personal care products, like toothpastes,^{82,83} lip balms,^{82,84} creams⁸⁴ and shampoos.⁸⁴ Aluminium oxide and silver nanoparticles in toothpastes⁸³ and moisturizing creams,⁵³ respectively, have also been measured by SP-ICP-MS, as well as plastic microparticles added as abrasives in exfoliant creams.⁸⁵ Sample preparation of cosmetics always requires their dispersion in ethanol or water, in most cases by adding a surfactant, although a previous defatting step with hexane is typical for sunscreens and lip balms.^{79,82,84} When refractory nanoparticles like TiO₂ or Al₂O₃ are involved, a previous matrix digestion with hydrogen peroxide has been applied.⁸³ Other consumer products considered have been antibacterial sprays containing silver nanoparticles,^{86–88} although the occurrence of other elements like Sn or Zn in these products has also been checked.⁸⁸

TiO₂ and metallic Ag are approved food additives, labelled as E171 and E174, respectively. Raw samples of E171^{46,89} and E174⁴³ have been characterised by SP-ICP-MS in combination with other techniques to obtain information about the nanoparticulate fraction in these materials. In the case of steels,⁹⁰ the aim of the analysis was the characterisation of refractory particles of titanium and niobium carbonitrides, whereas in petroleum products (crude and fuel oil,⁵⁷ and asphaltene solutions⁹¹) was the detection of iron and molybdenum containing particles. In a similar way, tattoo inks^{92,93} were analysed to study the occurrence of nanoparticulate Al, Ti, Cu, Cr, Zn and Pb from the pigments used in their fabrication, or the presence of copper nanoparticles in homeopathic medicines.⁹⁴

Given the nature of the materials considered and their commercial purpose, many of the studies were placed under the European Commission regulation and its definition of nanomaterial.^{43,46,79,81,83,84,93} The principal objective of the use of SP-ICP-MS was therefore focused on the characterisation of the nanoparticle fraction, being the mean size and the particle size distribution the main measurands of concern.

Although concentration is not a serious constrain in this scenario, a common limitation when using SP-ICP-MS is the detection of the smallest fraction of the nanoparticles present with these samples. This can lead to partial histograms since complete size distributions are not always obtained. This was

Table 2 Scenario 0: analysis of pristine nanoparticles

Sample	Nanoparticle composition	Sample preparation	Dwell time	Mass analyser	Collision/ reaction gas	Analytical information	Measurands	Complementary techniques	Ref.
Suspension	Ag	—	5 ms	Q	No	Qualitative (NP)	NP size distribution	TEM DLS	60
	Ag	—	100 µs	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP mass concentration DE mass concentration	TEM DLS	61
	Au@Ag (Ag shell)	—	50 µs	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration	AF4-UV-Vis	62
	Au-Ag alloy	—	6 ms 20 µs	Q	No	Characterisation (NP)	molar ratios Au : Ag	—	63
	Au	—	100 µs	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP number concentration	TEM	64
	Au (NP and nanorods)	—	6 ms 20 µs	Q	No	Characterisation (NP)	NP mean size	TEM DLS UV-Vis	65
	Au/polymer composite	—	100 µs	Q	No	Characterisation (NP)	Mean mass per NP NP mass distribution	ICP-MS TEM AF4-ICP-MS CFFF-ICP-MS	66
	CuO FeOOH	—	50 µs	DF	No	Characterisation (NP)	NP mean size NP size distribution	SEM XRD DLS	67
	Ni	—	500 µs	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP number concentration NP mass concentration DE mass concentration	SEM TEM XRD	68
	Pd	—	50 µs	Q	No	Characterisation (NP)	NP mean size NP size distribution	SEM SAXS XRD	69
	NaYF ₄ (Yb doped) NaYF ₄ (Er doped) NaGdF ₄ (Yb doped) NaGdF ₄ (Er doped)	—	100 µs	Q	No	Characterisation (NP)	NP size distribution	TEM XRD	70

Table 2 (Contd.)

Sample	Nanoparticle composition	Sample preparation	Dwell time	Mass analyser	Collision/ reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
Powder	TiO ₂	Dispersion in 2% PVP	50 µs	Q	No	Characterisation (NP)	NP mean size	DLS	71
						Quantitative (DE)	NP size distribution DE mass concentration	TEM	
	SiO ₂ /Pt	Dispersion	10 ms	Q	He	Characterisation (NP)	Mean mass per NP	ICP-MS SEM-EDS TEM XPS	72
						Qualitative (NP) Quantitative (NP)	— CNT number concentration	SEM-EDS NTA	73
	CNT (trace Y)	Dispersion in 1% Na deoxycholate	100 µs	Q	No	Qualitative (NP) Quantitative (NP)	— CNT mass concentration	ICP-MS TEM NTA UV-Vis	74
						Characterisation (NP)	Mean mass per NP NP mass distribution	SEM TEM SAM AES XPS XRD Raman	75
	TiO ₂ (Mn)	—	100 µs	Q	No				
Filters	Au	Water extraction	100 µs	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP mass concentration	HIM SEM-EDS XRD UV-Vis	76

Table 3 Scenario 1: analysis consumer and industrial products containing nanoparticles

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision /reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
Cosmetics Sunscreen	TiO ₂	Dispersion in ethanol	50 µs	DF	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration	—	77
	TiO ₂	Dispersion in 1% Triton X-100	100 µs	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration	ICP-MS SEM-EDS	78
	TiO ₂	Defatting with hexane + filtration	100 µs	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP number concentration DE mass concentration	ICP-MS SEM TEM DLS AF4-MALS-ICP-MS	79
Lip balm	TiO ₂ ZnO	Dispersion in 1% Triton X-100	—	—	—	Characterisation (NP)	NP mean size	TEM-EDS XRD	80
	TiO ₂ ZnO	Dispersion in 1% Triton X-100	5 ms	Q	He	Characterisation (NP) Quantitative (NP)	NP mean size NP number concentration NP mass concentration	AF4-MALS-ICP-MS	81
	TiO ₂	Defatting with hexane + filtration	100 µs	Q	He	Characterisation (NP)	NP size distribution	ICP-MS CE-ICP-MS	82
Toothpaste	TiO ₂	Dispersion in 0.1% SDS	100 µs	Q	He	Characterisation (NP)	NP size distribution	ICP-MS CE-ICP-MS	82
	TiO ₂ Al ₂ O ₃	H ₂ O ₂ digestion + dilution in 0.1% SDS	10 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration	ICP-MS STEM-EDS DLS AF4-MALS-ICP-MS HDC-ICP-MS	83

Table 3 (Contd.)

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision /reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
Sunscreen	TiO ₂	Defatting with hexane	100 µs	Q	No	Characterisation (NP)	NP mean size	ICP-MS	84
Lip balm		Dispersion in 0.1% SDS				Quantitative (NP)	NP size distribution NP number concentration	ICP-OES DLS	
Toothpaste						Quantitative (DE)	DE mass concentration		
Creams									
Shampoo									
Moisturizing cream	Ag	Dispersion in methanol	100 µs	Q	No	Characterisation (NP)	NP mean size NP size distribution NP number concentration NP mass concentration	ICP-MS FESEM TEM	53
Exfoliant	Plastic polymers (C-)	Dispersion in water	200 µs	Q	No	Qualitative (NP)	—	FESEM	85
Other consumer products									
Antibacterial spray	Ag	—	3 ms	Q	No	Qualitative (NP) Qualitative (DE)	—	HPLC-ICP-MS TEM	86
	Ag	—	10 ms	Q	No	Characterisation (NP)	NP mean size NP size distribution	ICP-MS	87
Commercial sprays	Ag-Sn-Zn	—	5 ms	Q	No	Characterisation (NP)	NP mean size	ICP-MS DLS SEM-EDS TEM-EDS SMPS	88
Food additives									
E174	Ag	Ethanol wetting + dispersion in 0.05% BSA	3 ms	Q	No	Characterisation (NP)	NP mean size	TEM	43
						Quantitative (NP)	NP size distribution NP number concentration NP mass concentration		

Table 3 (Contd.)

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision /reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
E171	TiO ₂	Dispersion in water	3 ms	Q	NH ₃	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration	TEM-EDS HAADF-STEM-EDS	46
Other materials Steel	TiO ₂	Dispersion in 0.05% BSA	3 ms	Q	No	Characterisation (NP)	NP mean size NP size distribution	SEM NTA AF4-MALS AF4-ICP-MS	89
	NbCN TiNbCN	Extraction 0.5 M H ₂ SO ₄ /0.1% Disperbyk-2012 + centrifugation	1.8 ms	TOF	No	Characterisation (NP)	NP size distribution Mass per NP distribution	STEM	90
	Fe ₃ O ₄	Dilution (<i>o</i> -xylene)	100 µs	Q	He	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP mass concentration DE mass concentration	ICP-OES TEM	57
Petroleum products									
Asphaltene solutions	Mo-Fe-	Dilution (<i>o</i> -xylene)	100 µs	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP mass concentration DE mass concentration	ICP-MS	91
Tattoo inks	Al ₂ O ₃ TiO ₂ Cu-	—	5 ms	Q	He	Characterisation (NP)	NP mean size NP size distribution	TEM DLS AF4-MALS	92
	Ti- Al- Cr- Cu- Zn- Pb-	—	5 ms	Q	He	Characterisation (NP) Quantitative (NP)	NP mean size NP number concentration NP mass concentration	ICP-MS	93
Homeopathic medicine	Cu	—	—	Q	—	Qualitative (NP)	—	DLS NTA SEM-EDS	94

^a M: nanoparticle with composition M. M-: nanoparticle containing element M.

the case for titanium in some cosmetics^{77–79,82} and food additives,⁴⁶ as well as for silver.^{43,53,86,87} For microplastics,⁸⁵ the sizes of detectable particles were restricted at both size range limits. At lower size range, it is restricted due to the inherent limitations of carbon detection by ICP-MS, and at the upper size range due to low nebulization efficiency of large particles, being limited to sizes from 1 up to 5 μm .

Scenario 2.1: laboratory tests involving nanoparticles or products containing nanoparticles

The applications developed under scenario 2.1, summarized in Table 4, include laboratory tests under controlled conditions, involving pristine nanoparticle or products containing nanoparticles. Laboratory tests with materials originally containing nanoparticles are devoted to studying their release into aqueous media under selected conditions, whereas the study of the fate and behaviour of nanoparticles in different media, or along different relevant processes, requires the addition of pristine nanoparticles to the samples of concern.

Release studies. The risk assessment of consumer and industrial products containing engineered nanoparticles involves knowing the human and environmental exposure to the nanoparticles released from such products along their whole life cycle. These release studies are usually performed at laboratory scale under controlled simulation conditions.

Direct human exposure to engineered nanoparticles and/or their transformation products might arise from the ingestion of foods stored in containers bearing nanoparticles (*e.g.*, Ag, nanoclays). Migration test from existing regulations for conventional analytes, based on the use of model food simulants (acetic acid, ethanol), have been applied to plastic food containers^{95–99} and packaging films,¹⁰⁰ as well as to cookware.¹⁰¹ The procedures are straightforward; after incubation of the samples at selected temperatures during fixed time periods, simulants are diluted conveniently and analysed by SP-ICP-MS. In addition to plastic containers, polymers doped with Fe_2O_3 ¹⁰⁹ and ZnO ¹¹⁰ have also been studied by using these migration tests. In the case of baby products containing silver nanoparticles, release studies were performed by leaching in artificial saliva to simulate the exposure route for children.⁹⁸ In a similar way, silver release from toothbrushes was studied by leaching in tap water.¹⁰² The dermal exposure from surfaces containing nanoparticles was simulated through wiping tests by using artificial sweat, followed by the extraction with water of the nanoparticles from the wipes.¹⁰³ Water extraction has also been applied to study the release of Ag nanoparticles from antibacterial plasters¹⁰⁴ or TiO_2 from textiles.¹⁰⁵

Environmental exposure to engineered nanoparticles from nano-enhanced products is related to their release and transformations that they undergo during their life cycle. Most commonly, materials like wood,¹¹⁶ leather¹¹⁷ or polymers^{108,109} have been subjected to direct leaching in water, whereas for paints^{111,113} or conductive inks,¹¹⁴ the release from painted surfaces or printed circuits have been considered, respectively. More realistic studies have involved the use of different model waters to study the leaching of metal bearing particles under

end-of-life conditions from photovoltaic cells,¹¹⁵ or the leaching of TiO_2 particles from painted surfaces by snow and rainwater under weathering conditions.¹¹² Mitrano *et al.*¹⁰⁶ studied the life cycle of nano-enhanced textiles subjected to different aging and washing processes to understand the release and transformation of the silver nanoparticles from the textiles. The release of metal containing particles from environmental samples, like road dust¹¹⁹ and mine tailings,¹²⁰ has also been studied by leaching in different media as a source of environmental pollution.

Regarding the composition of the nanoparticles, silver^{95–98,100,102–104,106,107,114,115,117} and titanium^{101,105,112,113} are the most frequently studied, although zinc¹¹⁰ and copper^{103,111,116} have also been considered. Multielement monitoring has also been performed, as in the case of photovoltaic cells,¹¹⁵ cookware¹⁰¹ or the emission of particles from paper printing and shredding.¹¹⁸ The release of carbon nanotubes from polymer nanocomposites was followed by monitoring the yttrium present in the nanotubes,¹⁰⁸ as it was described for pristine carbon nanotubes in scenario 0.^{73,74} Detection of plastic microparticles released from food containers has also been possible by using the carbon-13 isotope.⁸⁵

Although SP-ICP-MS allows to obtain detailed quantitative information about nanoparticle size and concentration, the technique has been used in some release studies only to confirm the presence of the nanoparticles in the food simulants^{85,95,99} or the leaching media,^{115,117,118} as well as of dissolved forms of the element monitored.⁹⁷ In these cases, the recorded time scans (Fig. 2a) allow to obtain the qualitative information required, once the adequate metrological criteria have been applied.⁴⁴ In many other studies, detailed information about mean/median sizes, size distributions and number/mass concentration of the nanoparticles released were reported, although the profiles of the size distributions revealed that the nanoparticle distribution had been partially recorded due to the attainable size limits of detection, hence underestimating the actual concentrations and overestimating mean sizes.

Fate studies. Whereas the release studies discussed above involve consumer and industrial products, fate studies are mostly based on the use of pristine and well-characterised nanoparticles, commercially available or synthesized at the laboratory, although aged nanoparticles have also been used.¹⁶⁴ The nanoparticles are spiked in the matrix of interest and the samples analysed directly or after undergoing the simulated process under study (*e.g.*, gastrointestinal digestion, laundry, wastewater treatment, environmental exposure). The main advantage of these controlled experiments is that both size and concentration of the spiked nanoparticles can be selected as desired. Although concentration is not a serious limitation for SP-ICP-MS, allowing even to work at realistic environmental concentrations,^{135,138,141} attainable size LODs can be a critical drawback when using SP-ICP-MS with real samples, as it has been discussed above and it will be shown again in relation to scenario 3.

Fate studies performed with spiked Ag nanoparticles in food simulants complement the migration tests of silver from food containers discussed above, allowing to obtain information

Table 4 Scenario 2.1: laboratory tests involving pristine nanoparticles or products containing nanoparticles

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/ reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
Release studies <i>Migration</i> Food containers	Ag-	Migration: - 3% acetic acid - 50% ethanol	100 μ s	Q	No	Qualitative (NP)	—	ICP-MS SEM-EDS	95
	Ag-	Migration: - 3% acetic acid - 10% ethanol	3 ms	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP mass concentration DE mass concentration	ICP-MS TEM-EDS	96
	Ag-	Migration: - Water - 3% acetic acid - 10% ethanol - 90% ethanol	10 ms	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP number concentration —	ICP-MS SEM-EDS	97
Food packaging films	Ag-	Migration: - Water - 3% acetic acid - 10% ethanol	3 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration	ICP-MS TEM-EDS	98
	Clays (Al-)	Migration: - 3% acetic acid - 10% ethanol	100 ms	Q	No	Qualitative (NP)	—	ICP-MS SEM-EDS	99
	Plastic polymers (C-)	Water	200 μ s	Q	No	Qualitative (NP)	—	FESEM	85
Cookware	Ag-	Migration: - Water - 3% acetic acid - 10% ethanol	5 ms	Q	No	Characterisation (NP)	NP mean size NP size distribution	ICP-MS STEM-EDS	100
	Al- Ti- Si-	Migration in 3% acetic acid	3 ms	Q	H ₂	Characterisation (NP) Quantitative (NP)	NP mean size NP number concentration	ICP-MS SEM-EDS	101
<i>Consumer products</i> Baby products	Ag-	Leaching in artificial saliva	3 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration	ICP-MS TEM-EDS	98

Table 4 (Contd.)

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/ reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
Toothbrush	Ag-	Leaching in tap water	3 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration	ICP-MS TEM-EDS	102
Dermal transfer from surfaces (keyboard and painted surface)	Ag-Cu-	Wiping test (artificial sweat) + water extraction	100 μ s	Q	No He	Characterisation (NP) Quantitative (NP)	NP mean size NP number concentration NP mass concentration	ICP-MS SEM-EDS TEM-EDS	103
Plaster	Ag-	Water extraction + filtration + CPE	10 ms	Q	No	Quantitative (NP)	Mass concentration NP	ICP-OES TXRF SEM-EDS	104
Textiles	TiO ₂	Leaching in water	100 μ s	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration	ICP-MS TEM-EDS	105
	Ag	Leaching under washing conditions	5 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP mass concentration	ICP-MS SEM TEM-EDS XANES	106
Washing machine effluent	Ag	—	4 ms 1 ms	DF	No	Characterisation (NP) Quantitative (NP)	NP mean size NP number concentration	ISE TEM-EDS NTA	107
<i>Industrial materials</i> CNT polymer nanocomposite	CNT (trace Y)	Leaching in water + surfactant stabilization	100 μ s	Q	No	Quantitative (NP)	NP number concentration	XPS ATR-FTIR FESEM	108
Pigmented polyethylene	Fe ₂ O ₃	Leaching in: - 0.05% SDS - Water - Hard EPA water - SRNOM Migration in: - Water - 3% acetic acid - 10% ethanol	5 ms	Q	H ₂	Characterisation (NP) Quantitative (NP)	NP mean size NP number concentration	ICP-MS TEM AUC FTIR	109

Table 4 (Contd.)

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/ reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
ZnO doped polymers	ZnO	Migration to: - Orange juice - Chicken meat - Tris-HCl extraction	3 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration	FAAS TEM-EDS FTIR DLS	110
Antifouling paint	Cu ₂ O	Leaching in freshwater from painted surface	100 µs	Q	No	Characterisation (NP)	NP size distribution	DLS XRD XPS SEM-EDS TEM-EDS	111
Paint	TiO ₂	Leaching in rainwater/snow from painted surface + filtration	50 µs	DF	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration	ICP-MS	112
	TiO ₂	Leaching in water from painted surface	100 µs	Q	No	—	—	SEM-EDS TEM HRTEM XRD UV-Vis XPS XRD	113
Printed circuits	Ag	Leaching in water	100 µs	Q	No	Characterisation (NP)	NP mean size	SEM AFM AF4-ICP-MS	114
Photovoltaic cells	Ag-Al- Cd-Mo-Se-Zn-	Leaching in: - Model freshwater - Model seawater - Model acidic rainwater	30 ms	Q	No	Qualitative (NP)	—	ICP-MS	115
Wood	Cu-	Leaching in water	—	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration	AAS ICP-OES SEM-EDS ATR-FTIR	116
Leather	Ag	Leaching in water	50 µs	Q	No	Qualitative (NP)	—	XPS FESEM	117

Table 4 (Contd.)

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/ reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
Paper	Multielement	Collection of airborne particles in filter + suspension in DMEM	—	Q	No	Qualitative (NP)	—	SEM-EDS TEM-EDS	118
<i>Environmental samples</i>									
Road dust	Pt-	Leaching in stormwater runoff	5 ms	Q	He	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP mass concentration	ICP-MS	119
Mine tailings	Th- U-	Leaching in different media + filtration	50 μ s	DF	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP number concentration DE mass concentration	ICP-MS	120
<i>Fate studies</i>									
<i>Food simulants</i>									
Water	Ag (spiked)	—	3 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP number concentration NP mass concentration	TEM AF4-MALS	121
Acetic acid (3%) Ethanol (10%)	Ag (spiked)	—	3 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP size distribution NP number concentration NP mass concentration DE mass concentration	ICP-MS	122
Water Acetic acid (3%) Ethanol (10–50%) Olive oil Milk									
<i>In vitro digestion</i>									
Gastric fluid	Au Ag ZnO CeO ₂ (spiked)	Gastric step	50 μ s	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP number concentration DE mass concentration	SEM	123
Digestion fluids	Ag (spiked)	3-steps <i>in vitro</i> -human gastro-intestinal digestion	—	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration	SEM-EDS TEM DLS	124
Chicken meat (spiked)	Ag (spiked)	3-steps <i>in vitro</i> -human gastro-intestinal digestion	10 ms	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP number concentration DE mass concentration	ICP-MS TEM-EDS	125

Table 4 (Contd.)

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/ reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
Orange juice	ZnO	3-steps <i>in vitro</i> -human gastro-intestinal digestion	3 ms	Q	No	Characterisation (NP)	NP mean size	FAAS	110
Chicken meat						Quantitative (NP)	NP size distribution NP number concentration NP mass concentration	TEM-EDS FTIR DLS	
<i>Laundry process</i>									
Washing solutions	Ag (spiked)	—	3 ms	Q	No	Characterisation (NP)	NP mean size	STEM-EDS	126
						Quantitative (NP)	NP size distribution		
						Quantitative (DE)	NP number concentration DE mass concentration		
<i>Waters</i>									
Synthetic moderately hard water + NOM	Pt (spiked)	—	50 μ s	Q	No	Characterisation (NP)	NP mean size	—	127
						Quantitative (NP)	NP size distribution NP number concentration		
	Au@Ag (spiked)	—	100 μ s	Q	No	Characterisation (NP)	NP mean size	—	128
						Quantitative (NP)	NP size distribution NP number concentration		
Ozonized water	Ag (spiked)	—	5 ms	Q	No	Characterisation (NP)	NP mean size	ICP-OES	129
						Quantitative (NP)	NP size distribution NP number concentration	DLS	
Lake water	Ag (spiked)	—	5 ms	Q	No	Characterisation (NP)	NP mean size	ICP-MS	130
						Quantitative (NP)	NP size distribution NP number concentration	AF4-UV-Vis	
	Ag (lake spike)	—	5 ms	Q	No	Characterisation (NP)	NP size distribution	ICP-MS	131
						Quantitative (NP)	NP mass concentration DE mass concentration		
	Ag (lake spike)	—	10 ms	Q	No	Characterisation (NP)	NP mean size	ICP-MS	132
						Quantitative (NP)	NP size distribution NP number concentration NP mass concentration	CPE + ICP-MS AF4-ICP-MS	
	Ag (lake spike)	—	50 μ s	DF	No	Characterisation (NP)	NP mean size	ICP-MS	133
						Quantitative (NP)	NP size distribution NP number concentration DE mass concentration	DGT + ICP-MS	

Table 4 (Contd.)

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/ reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
River water	Ag Ag ₂ S (spiked)	CPE	500 μ s	Q	He	Characterisation (NP)	NP mean size NP size distribution	HAADF-STEM-EDX	134
	Ag CeO ₂ Fe ₂ O ₃ (spiked)	Digestion (Na ₄ P ₂ O ₇)	50 μ s	Q	No	Characterisation (NP)	NP mean size NP size distribution	ICP-OES AF4-ICP-MS	135
	TiO ₂ ZnO (spiked)	—	100 μ s	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP number concentration NP mass concentration DE mass concentration	—	136
	Au Ag (spiked)	Fractions collected from HDC	3 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP number concentration	DLS AUC	137
Fresh waters	Ag	—	10 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP mass concentration	AF4-ICP-MS	138
Synthetic seawater	Au (spiked)	—	3 ms	Q	No	Characterisation (NP)	Size distribution	AF4-UV-Vis DLS	139
Seawater	Ag Ag ₂ S AgCl (spiked)	CPE	500 μ s	Q	He	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration	ICP-MS TEM-EDS	140
Seawater	Ag (spiked)	—	10 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP number concentration NP mass concentration	TEM	141
Seawater	Ag (spiked)	—	10 ms	Q	No	Characterisation (NP)	NP mean size NP size distribution	—	142

Table 4 (Contd.)

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/ reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
Waste water treatments Synthetic wastewater	Ag (spiked)	SPE (Chelex-100)	100 μ s	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP mass concentration	NTA	143
	TiO ₂ CeO ₂ (spiked)	—	—	Q	No	Qualitative (NP) Qualitative (DE)	—	ICP-MS	144
	Fe + adsorbed Cd(II)	—	3 ms	Q	H ₂ (MS/MS)	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP mass concentration	ICP-MS XRD	145
Wastewater	Ag (spiked)	—	500 μ s	Q	No	Characterisation (NP)	NP size distribution	EDM-HSI DLS TEM	146
Wastewater	Ag (spiked)	—	1 ms 100 μ s	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP number concentration DE mass concentration	TEM TOF-SIMS XPS UV-Vis	147
Wastewater	TiO ₂ Ag (spiked)	—	3 ms	Q	No	Characterisation (NP)	Average size	ICP-MS STEM-EDS	148
Wastewater River water	Au (spiked)	—	3 ms	Q	No	Characterisation (NP)	NP mean size NP size distribution	—	149
Wastewater River water	Ag (spiked)	Centrifugation	10 ms	Q	No	Characterisation (NP)	NP mean size NP size distribution	TEM-EDS DLS HDC-RI AF4-UV-Vis- MALS-RI HDC-ICP-MS	150
Activated sludge	CNT (trace Y) (spiked)	Centrifugation + ultrafiltration	10 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP number concentration	ICP-MS TEM-EDS	151
Activated sludge	Ag	Settling	100 μ s	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP mass concentration	—	152

Table 4 (Contd.)

Sample	Nanoparticle composition ^c	Sample preparation	Dwell time	Mass analyser	Collision/ reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
Biofilm reactor	Ag (spiked)	Settling	100 μ s	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP mass concentration DE mass concentration	ICP-MS TEM-EDS	153
Biofilm reactor	Ag (spiked)	Settling	100 μ s	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP mass concentration DE mass concentration	ICP-MS TEM-EDS	154
<i>Drinking water treatments</i>									
Fresh water	Ag Au TiO ₂ CeO ₂ ZnO (spiked)	—	100 μ s	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP mass concentration	TEM-EDS	155
<i>Soils and sediments</i>									
Soil	Ag ZnO TiO ₂ CeO ₂ (spiked)	Water extraction + centrifugation + filtration	10 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP mass concentration	SEM-EDS	156
	Ag (spiked)	Water extraction + filtration + CPE	10 ms	Q	No	Quantitative (NP)	Mass concentration NP	ICP-OES TXRF SEM-EDS	104
	Ag (spiked)	Water extraction + centrifugation + filtration Extraction (DTPA 5 mM) + centrifugation + filtration	10 ms	Q	No	Qualitative (NP)	—	ICP-OES	157
	Ag (spiked)	Water extraction + centrifugation + filtration Extraction (2.5 mM Na ₄ P ₂ O ₇)	50 μ s	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP number concentration NP mass concentration DE mass concentration	DLS TEM	158
	Ag (spiked)	Extraction (2.5 mM Na ₄ P ₂ O ₇) + settling	50 μ s	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP number concentration	ICP-MS	159
	Ag (spiked)	Water extraction + centrifugation + filtration	50 μ s	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP mass concentration	ICP-MS ISE TEM-EDS DLS	160

Table 4 (Contd.)

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/ reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
Soil colloids	CuO (spiked)	—	5 ms 100 μ s	Q	H ₂	Characterisation (NP) Quantitative (NP)	NP mean size NP number concentration	DLS	161
Soil sediment	Au (spiked)	Water extraction + centrifugation	10 ms	Q	No	Characterisation (NP)	NP mean size NP size distribution	ICP-MS AF4-MALS-UV- ICP-MS DLS	162
Soil sediment	Ag (spiked)	Extraction (2.5 mM Na ₄ P ₂ O ₇) + settling	3 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP number concentration	—	163
Sediment column	Aged Ag (spiked)	Extraction (different media) + centrifugation (+filtration)	5 ms	Q	No	Qualitative (NP)	(Presence of aggregates)	ICP-MS ICP-OES ETAAS TEM SEM-EDS HDC-ICP-MS	164
Sand column	Au (spiked)	—	3 ms	Q	No	Characterisation (NP)	NP size distribution	SEM	165
<i>Biological fluids</i> Artificial sweat	Ag (spiked)	—	100 μ s	Q	No	Characterisation (NP)	NP mean size	DLS UV-Vis XAS SEM-EDS	166
Plasma Cellular blood fractions	Fe ₃ O ₄ (spiked)	—	100 μ s	Q	H ₂	Characterisation (NP)	NP mean size NP size distribution	TEM AF4-UV-MALS- ICP-MS	167

^a M: nanoparticle with composition M. M-: nanoparticle containing element M.

about the oxidation of silver nanoparticles to dissolved forms.^{121,122} In relation to the potential human exposure to engineered nanoparticles through ingestion, the behaviours of Ag,^{123,124} but also of Au,¹²³ CeO₂¹²³ and ZnO^{110,123} nanoparticles along *in vitro* gastrointestinal digestions have been studied by SP-ICP-MS through the direct spiking of the digestion fluids^{123,124} or foods submitted to the *in vitro* digestions.^{110,125} With respect to the dermal exposure to consumer products containing Ag nanoparticles, laboratory experiments with artificial sweat have allowed to detect changes in size of the nanoparticles.¹⁶⁶ The stability of iron oxide nanoparticles in ferumoxytol, an intravenous preparation for treatment of the anaemia, was studied in human plasma and cellular blood fractions.¹⁶⁷ In relation to the life cycle of silver nano-enhanced textiles and their environmental implications, the fate and behaviour of pristine Ag nanoparticles in the washing solutions during the laundry process has also been studied.¹²⁶

Environmental scenarios for studying the fate of nanoparticles by SP-ICP-MS have included different types of real and synthetic surface waters (lake,^{130–133} river,^{134–137} sea^{140–142}) and wastewaters,^{143–150,275} as well as soils^{104,156–161} and sediments.^{162,163} Whereas spiked water samples were usually analysed directly or after dilution, soil analysis required the extraction and the separation of the nanoparticles from the soil matrix. This was done by using water or other extractants, like sodium pyrophosphate,^{158,159,163} followed by centrifugation or/and filtration.

Apart from works devoted to demonstrate the capability of SP-ICP-MS for detecting specific nanoparticles in water^{142,150} and soil^{159,161,163} samples, fate experiments focus mainly on studying the stability^{129,143} and transformations (dissolution/oxidation,^{128,138} aggregation^{127,135} ...) of nanoparticles under laboratory controlled conditions, although experiments adding nanoparticles into natural aquifers have also been performed.^{131–133} Special attention has been paid to the fate of nanoparticles in relation to drinking and wastewater treatments. The stability of Ag nanoparticles under ozonisation conditions¹²⁹ and the removal of different engineered nanoparticle through coagulation processes¹⁵⁵ and wastewater treatments^{151–154} have been studied. Soil studies have focused on the interaction of the nanoparticles with soil components, that control their transformations as well as their retention and mobility.^{157,162,164,165}

Most studies with environmental samples have focused on Ag nanoparticles,^{104,121–126,129–135,137–143,146–148,150,152–160,163,164,166,275} as well as their transformation products, Ag₂S and AgCl.^{134,140} Other nanoparticles studied include TiO₂,^{136,144,148,155,156} ZnO,^{136,155,156} CeO₂,^{135,144,155,156} CuO,¹⁶¹ Fe₂O₃,¹³⁵ Pt¹²⁷ and Au.^{137,149,155,165} Less common nanoparticles like core-shell Au@Ag nanoparticles¹²⁸ and carbon nanotubes¹⁵¹ have also been considered. The influence of the coating^{104,129,130,138,141,142,146,157,162,163} and the size^{123,126,149,150,155,159,163,165,166,275} of the spiked nanoparticles on the transformations studied have also been considered.

Fate studies focus on the transformations of the nanoparticles in relation to their potential toxicity.^{130–132,136,138,141,149,155,164} In general, the mean/median size and size distributions are the most frequent measurands. These measurands are related to the stability of the nanoparticles, but

also to their aggregation^{110,122–129,131,133,136,138,139,141,145,148,152,157,158,162–164,166} and dissolution.^{138,143,144,147,151} For this reason, the concentration of the dissolved element has also been considered in different studies.^{122,123,125,126,131,133,136,144,147,153,154,158} To confirm the formation of aggregates, electron microscopy techniques (SEM, TEM),^{110,123–125,141,147,151,158,164} DLS^{110,124,129,139,158,162,166} or NTA¹⁴³ have been used as complementary techniques.

Scenario 2.2: *in vitro*, *in vivo* and *ex vivo* (eco)toxicological tests

Risk assessment of nanomaterials must be based on exposure information but also on their toxicological behaviour in humans, animals and the environment. Hence, *in vitro*, *in vivo* and *ex vivo* (eco)toxicological essays worth studying in order to assess these behaviours and effects on living organisms. However, unravelling the mechanisms of action of nanoparticles for the correct interpretation of (eco)toxicity data requires the availability of detailed analytical information from characterising and quantifying nanoparticles and their transformation products in biological media.³⁴ In this context, SP-ICP-MS has proved to be a valuable technique in recent years, as it is summarized in Table 5. As in the case of the fate studies discussed above, (eco)toxicological test use mostly pristine and well-characterised nanoparticles, commercially available or synthesized at laboratory, allowing to select their size and coating, as well as their concentration levels.

***In vitro* studies.** Bacteria, fungi and algae have been micro-organisms used to assess the uptake, biotransformation and ecotoxicity of nanoparticles under laboratory-controlled conditions, in a similar way that it has been done with different types of cells for toxicity assessment.

Because many *in vitro* studies were performed with in-lab synthesised nanoparticles, SP-ICP-MS has been used in combination with other techniques only for characterisation of these pristine nanoparticles.^{168,169} The uptake of Te nanoparticles by *Staphylococcus aureus* and *Escherichia coli* was studied by the direct addition of spherical nanoparticles into the culture medium. Their biotransformation to nanorods when they were incorporated into the bacterial strains was confirmed by TEM and XRD, whereas SP-ICP-MS was applied after bacteria lysis to determine the number of particles per bacteria and to infer dimensional information of the rod-shaped Te nanoparticles.¹⁷⁰ The effect of TiO₂, ZnO and Ag nanoparticles on a river bacterial community was studied by mixing river water with artificial treated wastewater containing added nanoparticles. After three days of exposure, water samples were analysed by SP-ICP-MS to determine the mean size and size distributions, as well as the concentration of nanoparticles and dissolved species.¹⁷¹

Algae are commonly used in ecotoxicity testing. In this context, the dissolution of Ag nanoparticles and the bioavailability of silver to the model green algae *Chlamydomonas reinhardtii* was studied in a wastewater matrix,¹⁷² using SP-ICP-MS for monitoring the aggregation and dissolution of the nanoparticles. Speciation studies with chromium(III) and (VI) in ISO

Table 5 Scenario 2.2: *in vitro*, *in vivo* and *ex vivo* (eco)toxicological tests

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/ reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
<i>In vitro</i> studies									
<i>Fungi</i>									
<i>Aspergillus flavus</i>	Ag (spiked)	—	3 ms	Q	No	Characterisation (NP)	NP mean size NP size distribution	TEM DLS	168
<i>Aspergillus parasiticus</i>									
<i>Aspergillus carbonarius</i>						Quantitative (NP)	NP number concentration NP mass concentration		
<i>Aspergillus niger</i>									
<i>Aspergillus ochraceus</i>									
<i>Aspergillus steynii</i>									
<i>Aspergillus westerdijkiae</i>									
<i>Penicillium verrucosum</i>									
<i>Bacteria</i>									
<i>Pseudomonas aeruginosa</i>	Ag (spiked)	—	5 ms	Q	No	Characterisation (NP)	NP size distribution	TEM-EDS	169
<i>Staphylococcus aureus</i>						Quantitative (NP)	NP number concentration NP mass concentration	XRD FTIR	
<i>Staphylococcus aureus</i>	Te (internalized)	Bacteria lysis + centrifugation	5 ms	Q	No	Characterisation (NP)	NP mean size	TEM	170
<i>Escherichia coli</i>						Quantitative (NP)	NP size distribution NP number concentration	XRD	
River bacterial community	TiO ₂ ZnO Ag (spiked)	—	100 µs	Q	No	Characterisation (NP)	NP mean size NP size distribution NP number concentration NP mass concentration DE mass concentration	DLS	171
<i>Algae</i>									
<i>Chlamydomonas reinhardtii</i>	Ag (spiked)	Ultracentrifugation	0.5 ms	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP mass concentration DE mass concentration	ICP-MS	172
<i>Raphidocelis subcapitata</i>	Cr-(biosynthesis)	Filtration	50 µs	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP size distribution NP number concentration DE mass concentration	AAS TEM NTA	173
<i>Cells</i>									
Human umbilical vein endothelial cells	Au (spiked)	TMAH digestion	10 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP mass concentration	FIB-SEM Flow cytometry Confocal microscopy	174

Table 5 (Contd.)

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/ reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
Human macrophages and exosomes	Au (spiked)	Ultracentrifugation + lysis	5 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP number concentration	TEM NTA Flow cytometry Confocal microscopy	175
Human breast cancer cells	Au (spiked)	TMAH digestion	5 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP number concentration	ICP-MS	58
Human epithelial colorectal adenocarcinoma cells	Ag (spiked)	Lysis	3 ms	Q	No	Characterisation (NP)	NP mean size	ICP-MS	176
Human colon adenocarcinoma mucus secreting cells						Quantitative (NP) Quantitative (DE)	NP size distribution NP number concentration NP mass concentration	DLS TEM-EDS Confocal microscopy	
Mouse embryonic stem cells	Ag Ag ₂ S (spiked)	—	3 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration	ICP-MS TEM DLS	177
Mouse neuroblastoma cells	TiO ₂ Ag (spiked)	Lysis	3 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP number concentration	ICP-MS LA-ICP-MS TEM DLS	178
Trout liver cells	TiO ₂ (spiked)	Lysis	100 µs	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP number concentration NP mass concentration DE mass concentration	ICP-MS TEM-EDS DLS Confocal microscopy	179
<i>In vivo studies</i> <i>Plants</i> Tomato	Au (spiked)	Enzymatic digestion (macerozyme R-10)	100 µs	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP mass concentration DE mass concentration	TEM DLS	180
Tomato Pumpkin	CeO ₂ (spiked)	Enzymatic digestion (macerozyme R-10)	100 µs	Q	No	Characterisation (NP)	NP mean size NP size distribution NP mass concentration DE mass concentration	ICP-MS TEM	181

Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/ reaction cell gas	Analytical information	Measurands	Collision cell gas
Ag (spiked)	Enzymatic digestion (macerozyme R-10)	50 μs	Q	No	Quantitative (NP) Quantitative (DE)	NP number concentration DE mass concentration	IC TE
Pt (spiked)	Enzymatic digestion (macerozyme R-10) + filtration	100 μs	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP number concentration DE mass concentration	IC TE
Ag Zn InO (spiked)	Enzymatic digestion (macerozyme R-10)	3 ms	Q	No	Qualitative (NP)	—	IC SE TE DE
Ag ₂ S (spiked)	Enzymatic digestion (macerozyme R-10)	3 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP size distribution NP number concentration	DE SE TE XA
Pd (spiked)	Enzymatic digestion (macerozyme R-10)	100 μs	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration	IC TE
Ag (spiked)	Enzymatic digestion (macerozyme R-10)	10 ms	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP size distribution NP mass concentration DE mass concentration	IC XA
InO (spiked)	Enzymatic digestion (macerozyme R-10)	100 μs	Q	No	Characterisation (NP)	NP mean size	IC
CuO Cu(OH) ₂ (spiked)	Methanol extraction (50%)	100 μs	Q	No	Quantitative (NP) Quantitative (DE)	NP size distribution NP number concentration DE mass concentration	HI HI HI or
CuO (spiked)	Enzymatic digestion (macerozyme R-10)	100 μs	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration	IC TE
					Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP mass concentration DE mass concentration	FE TE

Table 5 (Contd.)

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/ reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
Wheat	Ag (spiked)	Enzymatic digestion (macerozyme R-10)	100 μ s	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP mass concentration DE mass concentration	ICP-MS TEM DLS	191
	Ag Au (spiked)	Enzymatic digestion (macerozyme R-10)	100 μ s	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP size distribution NP mass concentration DE mass concentration	TEM	192
Rice	TiO ₂ (spiked)	Enzymatic digestion (macerozyme R-10)	100 μ s	Q	No	Characterisation (NP) Quantitative (NP)	NP size distribution NP number concentration	ICP-OES DLS STEM-EDS	193
Rice Soybean	Ag (spiked)	Enzymatic digestion (macerozyme R-10)	50 μ s	Q	No	Characterisation (NP) Quantitative (DE)	NP size distribution DE mass concentration	TEM-EDS	194
Radish	CeO ₂ CuO (spiked)	Gastrointestinal digestion	100 μ s	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP mass concentration DE mass concentration	ICP-MS	195
	TiO ₂ (spiked)	Enzymatic digestion (macerozyme R-10)	100 μ s	Q	O ₂ /H ₂ (MS/MS)	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration	ICP-MS	196
	CeO ₂ (spiked)	Enzymatic digestion (macerozyme R-10) + filtration	100 μ s	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP mass concentration	ICP-MS LA-ICP-MS	197
<i>Soil organisms</i> <i>Lumbriculus variegatus</i>	Ag Au (spiked)	TMAH digestion	10 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration	ICP-MS	198
<i>Lumbriculus variegatus</i>	Ag (spiked)	Water extraction + centrifugation + filtration	—	Q	No	Characterisation (NP)	NP mean size NP size distribution	ICP-OES TEM DLS AF4-ICP-MS	199

Table 5 (Contd.)

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/ reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
<i>Lumbriculus rubellus</i>	Ag (spiked)	Enzymatic digestion (collagenase + hyaluronidase + proteinase K) + centrifugation	3 ms	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP number concentration NP mass concentration DE mass concentration	ICP-MS FESEM-EDS DLS	200
<i>Caenorhabditis elegans</i>	Au (spiked)	TMAH digestion	10 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP size distribution NP number concentration	ICP-MS SEM-EDS TEM DLS ICP-MS	201
<i>Aquatic organisms Daphnia magna</i>	Ag Au (spiked)	TMAH digestion	10 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration	ICP-MS	198
	Ag nanowire (spiked)	Collection of hemolymph	10 ms	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP number concentration NP mass concentration DE mass concentration	ICP-MS SEM TEM	202
Mussel	TiO ₂ (spiked)	Enzymatic digestion (proteinase K)	3 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP mass concentration	ICP-MS TEM	203
Zebra fish (liver, intestine, gills)	Au Ag (spiked)	TMAH digestion + filtration	1 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP mass concentration	TEM	204
Zebra fish (intestine, liver, gills, brain)	CeO ₂ TiO ₂ (spiked)	Enzymatic digestion (proteinase K) + H ₂ O ₂ digestion	3 ms	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP number concentration NP mass concentration DE mass concentration	ICP-MS TEM DLS	205
Trout (liver)	Ag (spiked)	TMAH digestion	3 ms	Q	He	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP number concentration NP mass concentration DE mass concentration	—	54

Table 5 (Contd.)

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/ reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
<i>Rat tissues</i> Spleen	Au (spiked)	TMAH digestion	3 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP mass concentration	—	206
	TiO ₂ (spiked)	Enzymatic digestion (proteinase K)	3 ms	Q	NH ₃	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP mass concentration	SEM-EDS TEM	207
Liver	Au (spiked)	Lysis + centrifugation	10 ms	Q	No	Characterisation (NP)	NP size distribution	TEM	208
	SiO ₂ (spiked)	Enzymatic digestion (proteinase K)	3 ms	Q	CH ₄	Characterisation (NP)	NP mean size	HPLC-ICP-MS ICP-MS TEM	209
Liver Lung	CeO ₂ TiO ₂ (spiked)	Enzymatic digestion (proteinase K)	3 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP size distribution NP mass concentration	ICP-MS DLS TEM	210
Lung Kidney Blood	TiO ₂ nanorods (spiked)	HCl/HNO ₃ digestion + filtration	—	Q	—	Qualitative (NP)		TEM SEM-EDS DLS	211
Foetus, resorption, placenta, lung, liver, spleen, kidney, mammary gland	Ag (spiked)	TMAH digestion	100 µs	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP number concentration NP mass concentration DE mass concentration	TEM-EDS	212
Stomach, intestinal content, liver, spleen, kidney, lungs, blood	Ag (spiked)	Enzymatic digestion (proteinase K)	3 ms	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP mass concentration DE mass concentration	AAS TEM DLS	213
<i>Chicken tissues</i> Liver Yolk	Ag (spiked)	Enzymatic digestion (proteinase K)	2 ms	DF	No	Characterisation (NP) Quantitative (NP)	NP size distribution NP number concentration NP mass concentration	AAS SEM-EDS	214
Ex vivo studies Human placenta	Ag (spiked)	Enzymatic digestion (proteinase K)	3 ms	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP number concentration NP mass concentration DE mass concentration	TEM DLS	215

^a M: nanoparticle with composition M. M-: nanoparticle containing element M.

Table 6 Scenario 3.1: analysis of foods

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analysers	Collision/reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
Confectionery									
Cakes	TiO ₂	H ₂ O ₂ digestion	3 ms	Q	No	Characterisation (NP)	NP size distribution	ICP-MS	216
Candy								AF4-ICP-MS	
Chewing gum									
Chewing gum	TiO ₂	Water extraction	10 ms	Q	NH ₃ (MS/MS)	Characterisation (NP) Quantitative (NP)	NP size distribution NP number concentration		217
Silver pearls	Ag	Water extraction	3 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration	ICP-MS TEM-EDS HAADF-STEM	218
Silver pearls									
Silver pearls	Ag	Water extraction + centrifugation	5 ms	Q	No	Qualitative (NP)		ICP-MS	44
Decoration dusting powder	Al Au								
Custard cream	TiO ₂		100 µs	Q	No	Qualitative (NP)		TEM-EDS CLS	219
Candies									
Candies	TiO ₂	Water extraction + filtration	100 µs	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP number concentration DE mass concentration	AF4-MALS-ICP-MS DLS	79
Candies									
Candies	TiO ₂	Centrifugation	100 µs	Q	No	Characterisation (NP)	NP size distribution	SEM	220
Silver coated chocolate and pearls	Ag	Ethanol wetting + dispersion in 0.05% BSA	3 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration	TEM	43

Table 6 (Contd.)

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analysers	Collision/reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
Meat									
Game meat	Pb	Enzymatic digestion (proteinase K)	5 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration DE mass concentration	ICP-MS	221
Miscellaneous									
Noodles	Al-	Enzymatic digestion (α -amylase)	3 ms	Q	No	Characterisation (NP) Quantitative (DE)	NP mean size NP size distribution DE mass concentration	ICP-MS	222
Drinks Chocolate Coffee Chewing gum Silver pearls	Ti- Si- Ag-	Water extraction + filtration	100 μ s	Q	He	Characterisation (NP) Quantitative (DE)	NP mean size DE mass concentration	ICP-MS DLS AF4-MALS-ICP-MS	223
Surimi sticks	TiO ₂	Enzymatic digestion (pancreatin and lipase)	100 μ s	Q	He	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP number concentration DE mass concentration	ICP-MS TEM	224

^a M: nanoparticle with composition M. M-: nanoparticle containing element M.

8692 algal medium showed the formation of Cr-bearing nanoparticles, detected by SP-ICP-MS, which could contribute to the ecotoxicity of chromium to *Raphidocelis subcapitata*.¹⁷³

The risk assessment of nanoparticles in living beings involves the proper evaluation of their behaviour at cellular level by *in vitro* assays. Within this framework, SP-ICP-MS has been used in a number of studies with human cell lines (umbilical vein endothelium,¹⁷⁴ macrophages and exosomes,¹⁷⁵ breast cancer,⁵⁸ human epithelial colorectal adenocarcinoma and colon adenocarcinoma mucus secreting cells¹⁷⁶) but also mouse (embryonic stem¹⁷⁷ and neuroblastoma¹⁷⁸) and liver trout¹⁷⁹ cells. The uptake of nanoparticles by cells was demonstrated to be dependent on their concentration in the media,¹⁷⁴ exposure time and cell line.⁵⁸ SP-ICP-MS allowed to study their biotransformations,¹⁷⁶ as well as their dissolution and agglomeration¹⁷⁶ within the cells. Moreover, the release of internalised nanoparticles by macrophages through exosomes could also be confirmed.¹⁷⁵

Au nanoparticles have been more frequently used in these *in vitro* studies with cells,^{58,174,175} although Ag,^{176–178} Ag₂S¹⁷⁷ and TiO₂ (ref. 178 and 179) have also been investigated. After exposure in the culture media containing the nanoparticles, cells were separated by centrifugation and lysed^{175,176,178,179} or digested with TMAH^{58,174} as a previous step to the analysis by SP-ICP-MS.

***In vivo* studies.** Release of engineered nanoparticles to the environment may end up and accumulate in edible plants, which is a potential pathway to human exposure. SP-ICP-MS has been used to study the uptake of nanoparticles by different types of plants in suitable growing media (hydroponic solutions^{185,187,192} and soils^{180–184,186,188,191,193–197}) containing known concentrations of nanoparticles of selected compositions and sizes. Edible plants like tomato, pumpkin, soybean, cucumber, thale cress, garden cress, white mustard, lettuce, kale, collard green, wheat, rice, soybean and radish have been selected for these experiments. Most of uptake studies have involved Ag nanoparticles,^{182,184,187,191,192,194} but also Ag₂S,¹⁸⁵ Cu,¹⁸⁴ CuO/Cu(OH)₂,^{189,190,195} ZnO,^{184,188} CeO₂,^{181,195,197} TiO₂,^{193,196} Au,^{180,192} Pd¹⁸⁶ and Pt.¹⁸³ By analysing different parts of the plants (roots, leaves, fruits), the uptake of nanoparticles through the roots and their translocation to other parts as intact particles or dissolved has been followed.^{183,196} In addition to the uptake of nanoparticles through the roots, the foliar exposure has been also assessed for Ag¹⁹⁴ and CuO^{189,190} nanoparticles. In the case of silver, the transformation of Ag nanoparticles after foliar exposure to ionic silver was reported.

In most cases, plant tissues were enzymatically digested by using macerozyme R-10, an enzyme mixture able to break down vegetal cell walls maintaining nanoparticles intact, as confirmed by several authors.^{182,190} To avoid dissolution of CuO/Cu(OH)₂ nanoparticles, Laughton *et al.*¹⁸⁹ proposed the use of methanol for extraction of the nanoparticles from lettuce leaves as an alternative to the enzymatic digestion.

The assessment of the nanoparticle ecotoxicity in aquatic and terrestrial environments is based on the use of model organisms. In this context, the bioaccumulation of Ag^{198–200} and Au^{198,201} nanoparticles in spiked sediments and soils has been

studied in the earthworms *Lumbriculus variegatus*,^{198,199} *Lumbriculus rubellus*²⁰⁰ and *Caenorhabditis elegans*.²⁰¹ Homogenates of the whole organisms were submitted to enzymatic²⁰⁰ or alkaline^{198,201} digestions, as well as to water extraction¹⁹⁹ prior to SP-ICP-MS analysis.

In relation to ecotoxicity studies in aquatic environments, the uptake of Ag^{198,202} and Au¹⁹⁸ nanoparticles by *Daphnia magna* was studied by analysing the hemolymph of the daphnids,²⁰² as well as whole organisms after alkaline digestion with TMAH.¹⁹⁸ In the case of mussels, Gallochio *et al.*²⁰³ suggested the *in vivo* formation of TiO₂ nanoparticles after their exposure to both ionic and particulate titanium. Zebra fish^{204,205} and trout⁵⁴ have also been used as target species for ecotoxicological studies with Ag, Au, TiO₂ and CeO₂ nanoparticles. The bioaccumulation of these nanoparticles was assessed by analysing different organs of the fishes (liver, intestine, gills, brain) by SP-ICP-MS after their digestion with TMAH or proteinase K.

Rats has been used extensively as model mammals for the toxicity assessment of nanomaterials. Different nanoparticles have been administered in a number of ways (oral gavage,^{207,213} intravenous injection,²⁰⁶ intratracheal instillation,²¹⁰ nose inhalation²¹² or intraperitoneal injection²⁰⁸) and their bioaccumulation and distribution in tissues were investigated by SP-ICP-MS. van der Zande *et al.*²¹³ reported the detection of silver nanoparticles in all the tissues studied after oral gavage, although the target organs were liver and spleen. Usually, samples were subjected to digestion with TMAH or proteinase K prior to SP-ICP-MS. Gallochio *et al.*²¹⁴ evaluated the oral administration of PVP-stabilized 20 nm Ag nanoparticles to chickens, detecting 16 nm nanoparticles in liver, but only ionic silver in egg yolks.

***Ex vivo* studies.** Experiments involving human tissues are scarce, nonetheless Vidmar *et al.*²¹⁵ conducted an *ex vivo* study involving human placentas obtained from pregnancies after caesarean section. The SP-ICP-MS analysis of the perfusion experiment confirmed the translocation and accumulation of Ag nanoparticles, although it could not be confirmed if the Ag-containing particles detected in the foetal circulation were translocated pristine Ag nanoparticles or Ag-bearing particles formed from dissolved silver that crossed the placental barrier.

Scenario 3.1: analysis of foods

A number of inorganic substances are approved as food additives by different regulations. Whereas metallic aluminium (E173), silver (E174) and gold (E175) are used to colour the external coating of confectionery, TiO₂ (E171) is added to provide a whitening effect, and SiO₂ (E551) as anticaking agent. All these additives have been re-evaluated recently by the European Food Safety Authority in relation to their safety, recommending that information about particle size and percentage (in number) of particles in the nanoscale should be included in their specifications when present in powder forms. Thus, as it has been seen in scenario 1 with E171 and E174 additives, regulatory requirements are the main driving factors for monitoring these elements by SP-ICP-MS, along with studying their occurrence as nanoparticles in foods. Table 6

Table 7 Scenario 3.2: analysis of environmental samples

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
Soils, sediments and sludges									
Soil	Fe-	Water extraction + centrifugation	3 ms	Q	NH ₃	Characterisation (NP)	NP mean size NP size distribution	ICP-MS NTA DLS TEM AF4-UV-ICP-MS	225
Mine tailings	FeAsO ₄ ·2H ₂ O (scorodite)	Water extraction + centrifugation	5 ms	Q	No	Characterisation (NP)	NP size distribution NP mass distribution	ICP-MS EXAFS XAS TEM-EDS	226
Lake sediment	Ag-	Water extraction + centrifugation + filtration	50 µs	DF	No	Characterisation (NP) Quantitative (NP) Quantitative (DE) Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP mass concentration DE mass concentration NP mean size NP size distribution NP number concentration NP mass concentration	ICP-MS	227
Road runoff sediment	Ag-Ti-	Extraction (TMAH)	50 µs	Q	No	Characterisation (NP)	NP mean size NP size distribution NP mass concentration DE mass concentration	ICP-MS	228
	Cu-Zn-Zr-Cd-Ce-Pt-Pb-	Surfactant extraction + centrifugation	5 ms	Q	H ₂ + He	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean mass NP size distribution NP mass distribution NP mass concentration DE mass concentration	ICP-MS	229
Sewage sludge	Ti-	Digestion (HNO ₃ + H ₂ O ₂) + filtration	3–10 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration	ICP-MS	230
	Ti-Fe-Zn-	Extraction (acetic acid) and centrifugation	100 µs	Q	n.r.	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP mass concentration DE mass concentration	ICP-MS FESEM-EDS TEM-EDS LDA	231
	Ti-	Extraction (sodium pyrophosphate) + centrifugation	—	TOF	No	Characterisation (NP) Quantitative (NP)	NP mass distribution NP number concentration	ICP-MS TEM	232

Table 7 (Contd.)

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
Waters									
Fresh waters	Ti-	Filtration	50 μ s	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration	ICP-MS TEM-EDS	56
	TiO ₂	Filtration	50 μ s	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP number concentration DE mass concentration	SEM-EDS FESEM	233
	Ti-	—	10 ms	Q	No	Quantitative (NP)	NP number concentration	ICP-MS ICP-OES SEM-EDS	234
	Ti-	Centrifugation	4 ms 3 ms	Q TOF	NH ₃ + He (MS/MS) No	Characterisation (NP) Quantitative (NP)	NP size distribution NP number concentration	TEM	235
	Ti-	—	50 μ s	DF	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration	—	77
	Ti-	—	3 ms	Q	No	Quantitative (NP)	NP number concentration	ICP-MS ICP-OES	236
	Ti-	Filtration	10 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP size distribution NP number concentration NP mass concentration DE mass concentration	CPE/TEM-EDS	237
	Ti-	Filtration	100 μ s	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration	ICP-MS ICP-AES TEM-EDS	238
	Ag-Ti-	—	50 μ s	Q	No	Characterisation Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration	HPLC-UV ICP-MS	228

Table 7 (Contd.)

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
	Ag	Filtration	50 μ s	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP number DE mass concentration	—	239
	Ag	Filtration	1 ms	DF	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP number NP mass concentration DE mass concentration	ICP-MS ICP-AES IC	240
	Ag	Cloud point extraction	100 μ s	Q	No	Characterisation (NP) Quantitative (NP)	NP size distribution NP mass concentration	ICP-MS ETAAS	241
	Ag-Ti-Ce	—	3 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number NP mass concentration	—	38
	Ag-Ti-Au	—	100 μ s	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP number NP mass concentration	FESEM-EDS	242
	Ce-Zn	—	100 μ s	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP number NP mass concentration	—	243
	Ti-Ce	Filtration	1 ms	DF	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP size distribution NP number NP mass concentration	ICP-MS ICP-AES FESEM-EDS IC	244

Table 7 (Contd.)

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
River water Rainwater	Ce- La-	—	50 μ s	DF TOF	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration DE mass concentration	—	245
	Zn-	Ion exchange (on line)	50 μ s	DF	No	Quantitative (DE) Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP mean mass NP mass distribution NP number concentration DE mass concentration	TEM-EDS	55
Seawater	Ag- Ti- Cu- Zn-	—	100 μ s	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration	ICP-MS	246
Sea water Fresh water	Ag- Ti- Ce-	Filtration	100 μ s	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration	—	247
Wastewater	Ag-	Filtration	5 μ s	Q	No	Characterisation (NP)	NP size distribution	ICP-MS ICP-OES	248
	Ag-	Settling	20 ms	Q	No	Quantitative (NP) Quantitative (DE)	NP mass concentration DE mass concentration	—	249
	Ag-	Filtration	100 μ s	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP mass concentration DE mass concentration	—	250
	Ag- Ti- Ce-	Filtration	100 μ s	DF	No	Quantitative (NP)	NP number concentration	—	251
Wastewater River water	Zn-	Cation exchange separation	0.5 ms	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP mass concentration DE mass concentration	—	252

Table 7 (Contd.)

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
Wastewater River water	Zn-	Settling and filtration	100 μ s 3 ms	Q TOF	No H ₂ + He	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration DE mass concentration	ICP-OES	253
Wastewater River water Lake water	Ag-	—	3 ms	Q	No	Quantitative (DE) Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP mass concentration	CPE + ETAAS	254
Tap water	Ag- Ti- Pb- Sn- Cu- Fe-	—	10 ms	Q	n.r.	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP size distribution NP mass concentration DE mass concentration	TEM-EDS	255
Various types of waters	Ag-	Cloud point extraction	100 μ s 0.5 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number concentration NP mass concentration DE mass concentration	ETAAS	256
Throughfall water	Cu-	—	100 μ s	Q	No	Characterisation (NP)	NP mean size	ICP-MS DLS AF4-MALS-ICP-MS	258
Acid mine drainage	Fe- Cu-	—	100 μ s	Q	NH ₃	Characterisation (NP) Quantitative (NP)	NP size distribution NP number concentration	ICP-OES STEM-EDS IC	259
Landfill leachates	Ti-	Filtration + surfactant stabilization (0.1% NovaChem 100)	3 ms	Q	NH ₃	Characterisation (NP) Quantitative (NP)	NP size distribution NP number concentration	ICP-OES SEM-EDS STEM-EDS DLS	260
Miscellaneous Cigarette smoke	As-	Electrostatic trapping	100 μ s	Q	No	Qualitative (NP)		ICP-MS HPLC-ICP-MS	261

Table 7 (Contd.)

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
Gas condensate	Hg-	Dilution with THF	100 μ s	Q	No	Characterisation (NP) Quantitative (NP) Quantitative (DE)	NP mean size NP size distribution NP number concentration NP mass concentration DE mass concentration	ICP-MS STEM-EDS AF4-UV-MALS-ICP-MS	262

^a M: nanoparticle with composition M. M-: nanoparticle containing element M. n.r.: not reported.

summarises the applications of SP-ICP-MS in relation with food analysis.

TiO₂ has been studied in different confectionery products, such as candies,^{79,216,219,220,223} chewing gum,^{216,217,223} pastry products²¹⁹ or cakes,²¹⁶ but also in drinks²²³ and surimi sticks.²²⁴ These samples were typically subjected to water extraction, followed by centrifugation or filtration,^{79,217,219,220} although Peters *et al.*²¹⁶ proposed the digestion with hydrogen peroxide to release the particles from the coating of the products. For the analysis of surimi sticks, samples underwent enzymatic digestion with pancreatin and lipase.²²⁴ The occurrence of particulate and dissolved aluminium in noodles was studied by Loeschner *et al.*,²²² after enzymatic digestion with α -amylase of the food products. Metallic silver, aluminium and gold are available as decorating powders or found as coatings of confectionery products.^{43,44,218} In these cases, samples had to be dispersed or extracted with water, or adding albumin as stabilising agent.⁴³ A different issue was addressed by Kollander *et al.*,²²¹ who studied the presence of Pb nanoparticles from ammunition in meat of hunted wild animals. Samples from the shot areas were digested with proteinase K and analysed by SP-ICP-MS, detecting Pb nanoparticles.

Although some analysis only focused on detecting the presence of nanoparticles in the samples,^{44,219} most of them covered their size characterization and the determination of number and mass concentrations as well. Moreover, most works included the determination of the total content of the elements under study in their analysis schemes, as well as considering the use of electron microscopy techniques^{43,218–220,224} or AF4 separations^{79,216,223} to verify or complement the results obtained by SP-ICP-MS.

Scenario 3.2: analysis of environmental samples

The widespread use of engineered nanomaterials in consumer products has increased their potential risk of environmental contamination, and consequently, the need and demand of analysis of environmental samples (waters, soils, sediments, sludges...) to provide reliable field information about their actual occurrence. On the other hand, SP-ICP-MS is also a suitable technique for monitoring naturally occurring or incidental nanomaterials.³² In fact, an still unresolved challenge in this scenario is the discrimination between engineered and natural occurring nanomaterials, which will be discussed below.

Table 7 summarizes different environmental samples analysed by SP-ICP-MS. Most cases refer only to the detection of nanoparticles containing the monitored element because the chemical composition of the particles was not available. Thus, the size information reported was based on assuming a specific composition (*e.g.*, metallic Ag, TiO₂) and a spherical shape. When the authors did not confirm the composition of the nanoparticles by an alternative technique, nanoparticles in Table 7 are referred as nanoparticles containing a specific element (*e.g.*, Ag-, Ti-, Ce- instead of Ag, TiO₂, CeO₂).

In most studies, total element concentrations were determined by ICP-MS or other atomic spectrometry techniques (ICP-OES, AAS) to obtain complementary information of the

samples. The use of complementary techniques, like TEM/FESEM or EXAF/XAS, for confirming the presence of nanoparticles or aggregates and obtaining information about morphology, size and composition of the particles was limited to samples containing particles at high enough concentrations.^{55,56,225,226,231–234,237,238,242,244,255,259}

Soils, wastes, sediments and sludges. Solid environmental samples analysed by SP-ICP-MS include soils,²²⁵ mine wastes,²²⁶ sediments^{227–229} and sludges from wastewater treatment plants.^{230–232} In the latter case, these analyses were complemented by the analysis of the influent and the treated effluent of the plants,^{230,231} or the analysis of the sediments of lake where discharges were carried out.²²⁹ The nanoparticles of interest were diverse, comprising particles containing elements such as Ag,^{227,228} As,²²⁶ Ti,^{228,230–232} Fe,^{225,231} Zn,^{229,231} Cu,²²⁹ Pb,²²⁹ Pt,²²⁹ Cd,²²⁹ Ce,²²⁹ or Zr.²²⁹ Sample preparation involved the extraction of the particulate matter with ultrapure water,^{225–227} the addition of surfactants²²⁹ or other reagents (sodium pyrophosphate,²³² acetic acid²³¹ or TMAH²²⁸); in the case of particles containing Ti, stronger procedures based on microwave acid digestion have also been applied.²³⁰ These treatments were followed by a separation step consisting of centrifugation, filtration, or both sequentially, prior to the analysis of the corresponding supernatant or filtrate.

In relation to the information reported, only Gomez-Gonzalez *et al.*²²⁶ determined by EXAFS and XAS the nature of the As-containing particles detected by SP-ICP-MS in mine tailings (FeAsO₄ · 2H₂O, scorodite), providing real size information confirmed by TEM. Due to the unknown nature of the particles detected, Baur *et al.*²²⁹ reported mean mass per particle and mass per particle distributions instead of information as equivalent size, which was the characterisation information typically reported, along with particle mass and number concentrations, as well as dissolved element concentrations.

Waters. Different types of waters have been analysed by SP-ICP-MS, including fresh waters from lakes, rivers and dams, as well as rain, sea, tap, pool and waste waters. The reasons for carrying out these analyses are diverse. The monitoring of titanium in river, lake and swimming pool waters is associated to the release of TiO₂ nanoparticles from sunscreens.^{234–238} Silver has been monitored in different types of waters, although the most relevant studies are related to the removal of silver containing particles in wastewater treatment plants,^{248–251,254} which has been also the case for Ti,²⁵¹ Zn^{252,253} and Ce-containing particles.²⁵¹ In the case of cerium, CeO₂ nanoparticles can be released into the air by diesel emissions being detected in rain water²⁴⁵ and other natural waters.^{38,243–245,247,251} Tap waters have been analysed in relation to the release of incidental nanoparticles containing different metals from copper pipes.^{255,256} Throughfall water from the wash-off of vine leaves treated with Cu-based fungicides has also been analysed by SP-ICP-MS in combination with other techniques to study the mobilization of Cu-containing particles.²⁵⁸ The occurrence of Zn,²⁴⁶ Ag,^{246,247} Ti,^{246,247} Cu²⁴⁶ and Ce²⁴⁷ containing particles⁴⁵ has also been monitored in seawaters. SP-ICP-MS was used to study acid mine drainage by monitoring Fe- and Cu-containing particles and the effects of water chemistry,²⁵⁹ as well as the

release of titanium from construction and demolition landfills by analysing their leachates.²⁶⁰

With respect to the preparation of water samples, the simplest procedures only involved the dilution of the sample, although settling, centrifugation or filtration were also applied when suspended matter was present.

In relation to the information reported, a reduced number of works provided qualitative information,^{236,248,258,274} relying on time scans (Fig. 2a) to prove the presence of particles, whereas most of articles provided quantitative information, including mean/median particle sizes, particle size distributions and particle mass and number concentrations, despite that partial size distributions were obtained in some cases.^{226,235,241,248,259} In this regard, although Rand and Ranville²⁵⁹ have demonstrated the utility of SP-ICP-MS for detecting incidental nanoparticles in natural systems, they also recommended caution in the data treatment and their interpretation.

In an attempt to discriminate between anthropogenic engineered nanoparticles from natural ones, the measurement of element ratios has been proposed. This strategy assumes that engineered nanoparticles are high purity substances, containing a single major element, while natural nanoparticles have heterogeneous multielement compositions. The Ce/La ratio has been reported for identification of CeO₂ nanoparticles,^{244,245,247} whereas for TiO₂ nanoparticles, the Ti/Al and Ti/V ratios have been considered.^{232,234–236,238,244,247} Whereas SP-ICP-MS with quadrupole instruments is limited to the measurement of two isotopes in an individual particle, TOF instruments are able to record the whole mass spectrum from each particle. In this respect, a promising approach for discrimination of anthropogenic and natural particles was proposed by Praetorius *et al.*²⁷⁶ based on the combination of single particle multielement analysis by SP-ICP-TOF-MS and machine learning data treatment.

Miscellaneous. The analysis of gaseous systems by SP-ICP-MS has been limited to condensates from cigarette smoke to study the presence of arsenic containing particles.²⁶¹ Condensates from cigarette smoke were accumulated in an electrostatic trapping device followed by washing with methanol. Whereas different inorganic and organoarsenic dissolved species were determined by HPLC-ICP-MS, no As containing particles were detected by SP-ICP-MS. Gas condensates from petroleum hydrocarbon samples were analysed by a number of techniques, including SP-ICP-MS, confirming the presence of Hg-containing nanoparticles by direct analysis of the samples diluted in THF.²⁶²

Scenario 3.3: analysis of biological samples

The potential exposure of living organisms, including humans, to engineered nanoparticles has led to study their occurrence in such biological systems. Other applications include the detection of wear metal particles from prosthesis in human fluids and tissues, as well as nanoparticles biosynthesised by microorganisms. Table 8 summarizes the applications under this scenario involving biological samples originally containing nanoparticles. As in scenario 3.2, in most works total element concentrations were determined by ICP-MS and also electron

Table 8 Scenario 3.3: analysis of biological samples

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/ reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
Microorganisms									
Yeast	Se	Enzymatic digestion (protease)	5 ms 100 µs	Q	H ₂	Characterisation (NP)	NP mean size NP size distribution	ICP-MS SEC-ICP-MS TEM-EDS	263
Aquatic organisms									
Molluscs	Ag	Enzymatic digestion (pancreatin/lipase)	50 µs	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number NP concentration	ICP-MS SEM	264
	Ti	Enzymatic digestion (pancreatin/lipase)	100 µs	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number NP concentration	ICP-MS DLS	265
	Ag-Ti-Cu-Zn	Enzymatic digestion (pancreatin/lipase)	100 µs	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number NP concentration NP mass NP concentration	ICP-MS	246
	20 elements	Alkaline digestion (TMAH) + filtration	3 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP mass NP concentration	ICP-MS	266
Various (plankton, crustaceans, molluscs, fish)	Ag-Ti	Alkaline digestion (TMAH)	50 µs	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number NP concentration NP mass NP concentration	ICP-MS	228
Human fluids and tissues									
Blood	Ag	—	5 ms	Q	No	Characterisation (NP)	NP mean hydrodynamic diameter NP mean mass per particle NP number NP concentration NP mass DE mass concentration	HDC-SP-ICP-MS µXRF µXANES	267

Table 8 (Contd.)

Sample	Nanoparticle composition ^a	Sample preparation	Dwell time	Mass analyser	Collision/ reaction cell gas	Analytical information	Measurands	Complementary techniques	Ref.
Urine	Ag-Ti	—	100 μ s	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number NP concentration	ICP-MS	268
Hip fluid	Co-Cr	Enzymatic digestion (proteinase K)	3 ms	Q	No	Characterisation (NP)	NP mean size NP size distribution	ICP-MS AF4-UV-MALS AF4-ICP-MS	269
Periprosthetic tissue	Ti-Ta-Co-Cr-Mo-Al-V	—	3 ms	Q	No	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP number NP concentration	ICP-MS SEM	270
Liver Spleen Kidney Intestine	TiO ₂ SiO ₂	Enzymatic digestion (proteinase K)	2 ms 100 μ s	DF Q	No H ₂	Characterisation (NP) Quantitative (NP)	NP mean size NP size distribution NP mass NP concentration	ICP-MS SEM-EDS	271
Liver Spleen	TiO ₂	Enzymatic digestion (proteinase K)	2 ms	DF	No	Characterisation (NP) Quantitative (NP)	NP size distribution NP number NP concentration NP mass NP concentration	ICP-MS SEM-EDS	272
Exhaled breath condensate	SiO ₂	—	3 ms	Q	He	Qualitative (NP)	—	TEM-EDS	273
Miscellaneous Gunshot residue wash from shooter's hand	Sb-Ba-Pb	Washing with water Swabbing + water extraction	30 μ s	Q	No	Qualitative (NP)	—	—	274

^a M: nanoparticle with composition M. M-: nanoparticle containing element M.

microscopy techniques were applied to obtain complementary information.

Microorganisms. Nanoparticles synthesized by microorganisms from dissolved precursors can be analysed by SP-ICP-MS in a similar way than those internalized from culture media once the microorganisms have been selectively digested, as it has been seen in scenario 2.2. This was the case of selenium-rich yeast,²⁶³ where biogenic-selenium nanoparticles were detected by SP-ICP-MS and confirmed by TEM-EDS, revealing the significance of nanoparticles in the speciation of metals and metalloids in biological systems.

Aquatic organisms. The analysis of aquatic organisms has included plankton,²²⁸ crustaceans,²²⁸ molluscs^{228,246,264–266} and fishes.²²⁸ In all cases, prior to SP-ICP-MS analysis, nanoparticles were extracted by digestion of the biological matrices. Enzymatic digestions included the use of pancreatin/lipase,^{246,264,265} whereas alkaline digestions were based on the use of TMAH.^{228,266} The most studied elements have been Ti^{228,246,265} and Ag,^{228,246,264} although nanoparticles containing Cu,²⁴⁶ Zn²⁴⁶ or different rare earth elements²⁶⁶ have been also considered. In relation to the information reported, all the articles provided quantitative information of particle mass and number concentration, particle size distribution and mean size. Reported size detection limits and size distributions allowed to confirm that nanoparticle distribution were incomplete in some cases.^{246,264,266} Moreover, and although SEM and DLS were used in some studies, these techniques did not provide conclusive information, hence the studies only confirmed the occurrence of particles containing the elements detected and not their specific nature.

Human body fluids and tissues. Although the monitoring of Ag-, Ti- and Si-bearing nanoparticles is supported by the potential exposure to Ag, TiO₂ and SiO₂ engineered nanoparticles,^{268,271,272} human fluids and tissues have also been analysed in relation with the release of incidental nanoparticles containing different elements from metal prosthesis^{269,270} and their migration from wound dressings.²⁶⁷ Moreover, exhaled breath condensates were analysed to detect the presence of respirable silica particles in quarry workers.²⁷³ In this latter work, the combination of SP-ICP-MS and TEM-EDS allowed to confirm the presence of silica and silicate particles in the breath condensates. Post-mortem tissues (liver, spleen, kidney, intestine) from deceased persons were analysed after enzymatic digestion with proteinase K, providing detailed information about the SiO₂ and TiO₂ found in the tissues and confirmed by SEM-EDS.^{271,272} On the other hand, periprosthetic tissues and hip fluids from arthroplasty patients were also analysed by SP-ICP-MS, confirming the presence of particles containing Co, Cr, Ti, V, Al, Ta and Mo and the importance of *in vivo* exposure assessments for realistic appraisal of metal toxicity and associated risks in arthroplasty.^{269,270}

Urine²⁶⁸ and blood²⁶⁷ samples were analysed directly after dilution with 1% glycerol or ultrapure water, respectively. Whereas particles containing Ti and Ag were detected in urine, only dissolved Ag was detected in blood from burned patients treated with wound dressings containing Ag nanoparticles.

SP-ICP-MS has also been used with forensic purposes for the direct analysis of gunshot residues.²⁷⁴ Residues were sampled from the hands of shooters by washing with ultrapure water or swabbing (followed by water extraction from the swabs) to obtain the nanoparticle suspensions, which were analysed directly for screening the presence of Sb-, Ba- and Pb-bearing particles.

Instrumentation and data acquisition

As explained above, SP-ICP-MS was originally developed in commercial quadrupole mass spectrometers with data acquisition frequencies in the range of 1–100 Hz, by using dwell times in the millisecond range. The implementation of single particle detection in quadrupole instruments by manufacturers implied that higher acquisition frequencies became feasible by working at dwell times in the microsecond range, removing the settling time of the quadrupole between readings and improving the capability for transmission and storage of data. Although this new generation of instruments was commercially available since 2014, Tables 2–8 show that both ranges of dwell times are being currently used, mostly depending on the availability of instruments, with 45% and 55% of applications using microsecond and millisecond dwell times, respectively. In any case, the duration of the particle events limits the selection of dwell times. Dwell times longer than twice the duration of the events are recommended¹⁷ when working at millisecond dwell times to record the particle events as pulses, whereas for microseconds, they should be shorter than half the duration of the events. Working at dwell times longer than 10 ms increases the number of events corresponding to two or more nanoparticles, whereas dwell times around the duration of a single nanoparticle event (300 μ s to 1 ms) makes difficult to confirm whether the recorded events correspond to one or more nanoparticles. In any case, an adequate dilution of the sample must be made, if needed, in accordance with the dwell time used, because it also affects to the number concentration linear range.¹³ In spite of these constraints, dwell times in the range of 0.5–2 ms have been used in several works, compromising the quality of their results.

The feasibility of using microsecond dwell times with double focusing ICP-MS also depends on the instrument, with last generation instruments capable of working down to 10 μ s.¹¹ In a similar way, a new design of TOF mass spectrometer with a temporal resolution of 33 μ s became commercially available recently.⁹ In either case, their less widespread use is reflected in the low number of applications found in Tables 2–8. The main feature of TOF instruments lies in their simultaneous multi-element capability, recording nearly the whole mass spectrum within each reading. Although applications of SP-TOF-ICP-MS are still scarce, the technique offers unique performance for multi-element and isotope ratio analysis in individual nanoparticles, and for discerning between naturally occurring and engineered nanoparticles.³² Although multielement SP-ICP-MS with quadrupole instruments has also been reported,²⁷⁷ it is limited to monitoring up to two isotopes, and is not commercially available yet.

Apart from resolving spectral interferences, double focusing instruments provide better transmission efficiency and hence improved sensitivity with respect to quadrupole and TOF instruments, resulting in the lowest available size LODs.⁷⁷ The lower resolution of quadrupole ICP-MS is compensated by using single quadrupole instruments with collision/reaction cells (see Tables 2–8), as well as multipole instruments in MS/MS mode,^{145,196,217,235} to reduce polyatomic interferences and improve size limits of detection for nanoparticles containing Ti, Fe, Si or Se. However, the collisions/interactions of the ion cloud generated by each particle with the collision/reaction gases increase the duration of the particle events, affecting the performance of the measurements.²⁷⁸ In fact, the use of short dwell times instead of collision/reaction cell technologies has been proposed as an alternative for improving size LODs for silica particles.²⁷⁹

Sample preparation

When ICP-MS is used in single particle mode, the preservation of most of the properties of the nanoparticles is mandatory, hence sample treatments should be reduced to the minimum required. Apart from the extraction of the nanoparticles from solid samples, sample treatments prior to SP-ICP-MS measurements have been limited to clean-up procedures for removal of large particles or fats, the separation of nanoparticles from dissolved species and the preconcentration of the formers.

As it has been shown in the different scenarios discussed above, extraction of nanoparticles from solid samples can involve just the use of water or aqueous solutions containing surfactants, although 50% methanol has been proposed to avoid the dissolution of copper oxide nanoparticles.¹⁸⁹ However, for more refractory nanoparticles, like TiO₂, SiO₂ or CeO₂, more aggressive treatments based on the use of strong acids and/or hydrogen peroxide have been applied.^{83,205,216,230} Nanoparticles from soils and sediments have been often extracted with tetrasodium pyrophosphate,^{158,159,163} commonly used to disperse soil heteroaggregates. On the other hand, biological samples require the degradation of the organic matrix by using alkaline reagents, like TMAH, or enzymes. In this regard, enzymatic digestions of plant samples have relied exclusively on the use of macerozyme R-10, a mixture of cellulase, hemicellulase and pectinase, whereas for animal samples, proteinase K has been most often selected, although also mixtures of pancreatin/lipase²²⁴ and amylase²²² have been used. In this regard, there is not a general agreement about the best digestion approach. For example, Loeschner *et al.*²⁰⁶ reported that both TMAH and proteinase K provided similar size distributions for gold nanoparticles in animal tissues, although mass recoveries with proteinase K were not quantitative. In contrast, it was shown that enzymatic treatment with proteinase K was more suitable for silver nanoparticles in human placental tissue, as TMAH treatment appeared to change the nanoparticles, most likely by silver ion precipitation and/or nanoparticle aggregation.²⁸⁰

Once nanoparticles have been extracted from a solid sample, it is usual to separate the liquid phase containing the nanoparticles from the solid residue to remove large particles that

may clog the nebulisers. This is also the case in release and fate studies involving solid materials as well as in the analysis of waters. Both filtration and centrifugation have been used for this purpose, although several authors have reported significant losses of nanoparticles in membrane filters.^{126,159,163,245} Thus, filtration should be discouraged unless quantitative recoveries have been proved, therefore settling or centrifugation should be used on a routine basis if removal of large particles is needed. Other clean-up procedures involved the defatting of cosmetics by using hexane.^{79,82,84}

The presence of dissolved species of the measured element has a negative effect on the size LODs, hence methods including their removal by ion exchange^{55,252} or chelating resins¹⁴³ prior to SP-ICP-MS measurements have been reported.

Nanoparticle concentration is not a serious limiting factor for SP-ICP-MS in most scenarios due to its low limits of detection down to 100 particles per millilitre. When lower LODs are required, cloud point extraction has proved to be a valid approach for the isolation and concentration of nanoparticles, while preserving their core size and morphology.^{104,132,140,241,256,281} Cloud point extraction involves the addition of a non-ionic surfactant (*e.g.*, Triton X114) at concentrations over the critical micellar concentration, the incorporation of the nanoparticles in the micellar aggregates and the separation of the surfactant phase from the aqueous one by mild heating (*ca.* 40 °C). Besides the addition of a complexing agent allows the selective extraction of the nanoparticles in the presence of the corresponding cations.²⁸² Finally, a further dilution of the surfactant phase in a mixture of ethanol and water is required before SP-ICP-MS measurements.

As it has been discussed in the previous section, nanoparticle concentration of the measured suspensions should be low enough to be within the linear range, below 10⁷–10⁸ L^{−1} depending on the instrumental and acquisition conditions,¹³ and hence the adequate dilution of the suspensions must be considered in each case.

Separation techniques coupled to SP-ICP-MS

Separation techniques like asymmetrical flow and centrifugal field flow fractionation, capillary electrophoresis, differential mobility analysis, hydrodynamic chromatography, as well as other chromatography modes (size exclusion, reverse phase, ion exchange) have been coupled to ICP-MS. In this situation, the ICP-MS instrument acts as an element specific detector of inorganic nanoparticles that are separated according to their size, density, surface properties or charge.²⁸³ However, the coupling of an ICP-MS working in single particle mode allows to obtain additional information related to the homo/hetero-aggregation/agglomeration of primary inorganic nanoparticles as well as the element content in complex nanoparticles or nanocomposites, in addition to the information obtained directly through the separation itself.

Although methods based on the online coupling of SP-ICP-MS to HDC,²⁸⁴ AF4,²⁸⁵ capillary electrophoresis²⁸⁶ and DMA²⁸⁷

have been reported since 2012, most of them must be considered as proofs-of-concept because their applications to complex scenarios are still scarce or absent, as in the case of capillary electrophoresis and DMA.

The online coupling of HDC to SP-ICP-MS was first described by Pergantis *et al.*²⁸⁴ for the simultaneous determination of nanoparticle size, number concentration and metal content, by using pristine Au nanoparticles. Subsequently, the capability of HDC-SP-ICP-MS to identify and characterize nanoparticle homoagglomerates in complex media by determining the mass and the hydrodynamic diameter of the separated particles was also demonstrated.²⁸⁸

Roman *et al.*²⁶⁷ developed an algorithm to deconvolute the SP-ICP-MS signals of dissolved element and nanoparticles separated by HDC, providing information about the concentration of dissolved silver and the distribution of Ag nanoparticles in terms of hydrodynamic diameter, mass-derived diameter, number and mass concentration. The approach was suitable to study quantitatively the dynamics and kinetics of silver nanoparticles in complex biological fluids, including processes such as agglomeration, dissolution and formation of protein coronas. The method was applied to investigate the presence of Ag nanoparticles in the blood of burn patients treated with silver dressings, although only dissolved species were detected.

Under less complex conditions, HDC-SP-ICP-MS has been successfully applied to simultaneously determine both the hydrodynamic radius and the content of Au nanoparticles in liposomes used as carriers of Au nanoparticles.²⁸⁹ It was possible to distinguish between subpopulations of liposomes with different hydrodynamic diameters and various nanoparticle loads. The application of HDC-SP-ICP-MS to the analysis of river and wastewaters under optimized conditions did not allow to detect silver nanoparticles in the samples, although Cu-containing nanoparticles could be identified.²⁷⁵

The feasibility of using AF4 online coupled with SP-ICP-MS to detect and quantify inorganic nanoparticles at environmentally relevant concentrations was firstly investigated by Huynh *et al.*²⁸⁵ by using Ag and Ag-SiO₂ core shell nanoparticles. Later on, Hetzer *et al.*¹⁰⁰ used AF4-SP-ICP-MS to evaluate the migration behaviour of Ag nanoparticles from food packaging films with varying nanosilver content into three different food simulants (water, 3% acetic acid and 10% ethanol), verifying that both silver nanoparticles and silver nanoparticles/polymer heteroaggregates were released in water.

An alternative to online couplings is the analysis of the fractions collected from the effluents of HDC or AF4 by SP-ICP-MS. This was the approach followed by Proulx *et al.*,¹³⁷ who demonstrated the feasibility of using HDC offline coupled to SP-ICP-MS for detecting of 20 nm Ag nanoparticles spiked in a river water sample, as well as by Woo-Chun *et al.*²⁹⁰ who analysed tap, river and waste waters spiked with 30, 60 and 100 nm silver nanoparticles. However, the nanoparticle concentrations used in both studies, in the range of mg L⁻¹, were well above those expected in the environment. In a simpler scenario, the content of metal nanoparticles in composite particles consisting of Au nanoparticles embedded in polymeric particles was determined by using both asymmetrical flow and centrifugal field-flow

fractionation.⁶⁶ Nanoplastics were separated according to their hydrodynamic diameter and buoyant mass, respectively, and the collected fractions analysed by SP-ICP-MS, confirming the presence of between 1 and more than 8 Au nanoparticles per plastic particle. Under more complex conditions, the SP-ICP-MS analysis of fractions collected from AF4 separations allowed to obtain complementary information about Ag nanoparticles in chicken meat digestates²⁹¹ and Al₂O₃ and TiO₂ nanoparticles in toothpaste.⁸³

Techniques complementing SP-ICP-MS: analytical platforms

As it has been highlighted through the previous sections, SP-ICP-MS has opened the way for analysing a variety of samples in different scenarios, allowing the detection of nanoparticles, the determination of their concentrations and their characterization to a certain level. However, SP-ICP-MS shows intrinsic limitations, mainly with respect to morphological characterization (size, shape, aggregation/agglomeration) and composition, therefore SP-ICP-MS is usually complemented by using additional techniques (see column “Complementary techniques” in Tables 2–8), leading to advanced analytical platforms which are required for solving complex analytical problems. In the end, the size information provided by SP-ICP-MS has to be estimated from the content of the element directly measured in the particles together with their shape and composition, which must be determined by other complementary techniques; otherwise, these morphological parameters must be assumed and only an equivalent size could be reported. The first purpose of these complementary techniques is to provide additional information on nanoparticle size and shape, to supplement or validate the SP-ICP-MS information. In this way, dynamic light scattering, nanoparticle tracking analysis, and particularly electron microscopy are the most common techniques.

Due to its high spatial resolution, below 1 nm, TEM is one of the most powerful techniques to visualize nanoparticles, and then to obtain information not only about their size, but also shape and aggregation state. This technique is essential in the characterization of pristine nanoparticles (scenario 0), to verify the success of the synthesis of new nanoparticles and to confirm their properties. When more complex analytical scenarios are considered, SEM often substitutes TEM. Current SEM instruments, working with field-emission electron sources (FESEM), offer improved spatial resolutions, reaching competitive ranges for the determination of nanoparticle size and shape in complex samples with easier sample preparation than TEM. Moreover, FESEM provides images of larger sample areas, obtaining more representative size distributions. In any case, electron microscopy plays a significant role to evaluate shapes and possible aggregations of nanoparticles in scenarios 1 and 2, and even in scenario 3 if concentrations of nanoparticles are high enough.

On the other hand, unlike light scattering techniques, electron microscopy enables the determination of the chemical composition of the nanoparticles. Most SEM and TEM instruments include several detectors and are usually coupled to EDS,

obtaining elemental composition of the nanoparticles for their identification, allowing the verification of their nature, but also studying their reactivity, stability and transformations. Although EDS is frequently coupled to TEM or SEM in any analytical scenario, other techniques like electron diffraction and electron energy-loss spectroscopy have also been considered to obtain structural information. The use of transmission electron microscopes working in scanning mode (STEM),^{83,90,100,126,148,193,259,260,262} with high-angle annular dark-field detection,^{46,134,218} allows to obtain Z-contrast images that can also add information about the chemical composition of the nanoparticles.

Even though XAS techniques do not provide information on nanoparticle morphology, they have also been considered in some works^{166,182,185,187,226} because of the interest for improving the information about the composition of the nanoparticles in complex samples. Whereas XANES has been used to obtain information on the geometry and oxidation state of the elements,^{106,267} EXAFS has done it on element coordination.²²⁶ However, these techniques have been applied occasionally because of their limitations in sensitivity, data interpretation and availability of synchrotron radiation facilities. XRD is also a technique to be considered to obtain structural information of nanoparticles, although limited to pristine nanoparticles (scenario 0) or scenarios in which nanoparticle concentration is not a limitation (scenarios 1 and 2). Finally, the determination of the total element content in the samples under study is included in most works, to evaluate mass balances and recoveries, by using atomic spectrometry techniques (ICP-MS, ICP-OES, AAS) after a suitable digestion step.

SP-ICP-MS based immunoassay and hybridization methods

ICP-MS-based immunoassay methods for determination of biomolecules has gained increasing interest in recent years.²⁹² Immunoassays are based on the use of an antibody (or antigen) as a biorecognition agent of the analyte of interest, which acts as antigen (or antibody), respectively. Immunoassays are very widely used in clinical diagnostics, environmental and food safety, covering a range of analytes from small molecules to macromolecules. The high selectivity and affinity of an antibody against its antigen allows their specific binding in the presence of complex matrices (*e.g.*, whole blood, serum, urine, foods). Most immunoassays require the labelling of the antibody (or the antigen) with easily detectable markers (*e.g.*, radioisotopes, enzymes, small molecule light absorbers, fluorophores, nanoparticles). ICP-MS immunoassays are based on the use of elemental tags of metal ions, nanoparticles or metal containing polymers. Although element-tagged immunoassays were firstly proposed in the 1970s,²⁹³ it was not until the advent of ICP-MS that this methodology started to be relevant, largely due to its higher sensitivity in comparison with other atomic spectrometry techniques and its multiplexing capability.^{294,295}

The use of metal nanoparticles as labels provides significant advantages, owing to their stability, biocompatibility, and easy

conjugation to antibodies/antigens without modifying their binding properties. Moreover, when used in combination with an ICP-MS operated in single particle detection mode a further improvement in sensitivity is achieved. Whereas ICP-MS immunoassays are based on the conventional quantification of the tagging element, reaching detection limits in the range of 50–5000 pg mL⁻¹, these detection limits can be decreased to 1–15 pg mL⁻¹ when SP-ICP-MS is used.²⁹² The improvement lies in the fact that the biomolecule concentration is related to the number concentration of nanoparticles determined by SP-ICP-MS and not to the total content of element measured by ICP-MS.

The first immunoassay based on SP-ICP-MS was reported in 2009. α -Fetoprotein was determined by a competitive immunoassay, using 45 nm Au nanoparticles as labels. A detection limit of 16 pg mL⁻¹ was achieved, lower than using other immunoassay strategies.²⁹⁶ Rabbit-anti-human immunoglobulin G was determined by a sandwich type immunoassay using 45 nm Au nanoparticles with detection limits of 100 pg mL⁻¹.²⁹⁷ A self-validated homogeneous immunoassay was also proposed for the carcinoembryonic antigen quantification by monitoring both frequency and intensity of the gold nanoparticles used as labels. The method provided accurate results in human serum samples with detection limits in the pM level.²⁹⁸ ZnSe quantum dots were used for tagging antibodies in a sandwich-type magnetic immunoassay for determination of carcinoembryonic antigen in human serum with a detection limit of 6 pg mL⁻¹ by monitoring ⁶⁴Zn.²⁹⁹ The determination of cytokeratin fragment antigen 21-1, carbohydrate antigen, and carcinoembryonic antigen was carried out by a simultaneous sandwich-type immunoassay using antibody-immobilized magnetic beads and Au, Ag and ZnSe nanoparticle labels, respectively.³⁰⁰ The method was successfully applied to detect the three biomarkers in human lung cancer serum samples.

Besides immunoassay, SP-ICP-MS has also been applied in DNA hybridization and RNA methods.^{301–304} A homogeneous DNA assay based on a target-induced hybridization chain reaction to achieve controlled spherical nucleic acid assembly has been reported. The strategy relies on the mediation of the hybridization chain reaction in the assembly of a nanogold core with oligonucleotide shell to generate controllable large Au nanoparticle aggregates and significant ¹⁹⁷Au counts as compared with the background of a simple dispersed Au nanoparticle. This homogeneous assay could determine DNA within the range of 5 fM to 10 pM.³⁰²

A rRNA detection platform was achieved by combining a sandwich type hybridization reaction with a single-molecule magnetic capture and SP-ICP-MS for the absolute and relative quantification of *E. coli* rRNA. This method was applied to the direct quantification of rRNA from dangerous human pathogens in milk samples with a detection limit of 10 fM.³⁰⁴

Table 9 summarises the SP-ICP-MS based immunoassay and hybridization methods developed up to date. Au nanoparticles have been more frequently used, although methods based on Ag and Pt nanoparticles have also been reported, as well as ZnS quantum dots. Quadrupole instruments have been used in all

Table 9 SP-ICP-MS-based immunoassay and hybridization methods for the quantification of biomolecules

Analyte	Sample	Assay type	Nanoparticle label	Dwell time	LOD	Ref.
α -Fetoprotein	Human serum	Competitive immunoassay	Au (45 nm)	10 ms	16 pg mL ⁻¹	296
Rabbit-anti-human IgG	Human serum	Sandwich immunoassay	Au (45 nm)	10 ms	100 pg mL ⁻¹	297
Carcinoembryonic antigen	Human serum	Homogeneous immunoassay	Au (30 nm)	50 μ s	210 pg mL ⁻¹ (1.2 pM)	298
	Human serum	Sandwich magnetic immunoassay	ZnSe (2.6 nm)	100 μ s	6 pg mL ⁻¹	299
Cytokeratin fragment antigen	Human serum	Sandwich magnetic immunoassay	Au (29 nm)	100 μ s	20 pg mL ⁻¹	300
Carcinoembryonic antigen			ZnSe (2.6 nm)		6 pg mL ⁻¹	
Carbohydrate antigen			Ag (14 nm)		0.25 mU mL ⁻¹	
DNA	—	Homogeneous DNA hybridization	Au (28 nm)	0.5 ms	1 pM	301
	Human serum	Homogeneous DNA hybridization chain reaction	Au (30 nm)	5 ms	3 fM	302
Human immunodeficiency virus	Human serum	Multiplexed heterogeneous sandwich DNA hybridization	Au (25 nm)	0.5 ms	<1 pM	303
Hepatitis B virus			Ag (25 nm)			
Hepatitis C virus			Pt (20 nm)			
<i>E. coli</i> RNA	Milk	Sandwich hybridization with magnetic particles	Au (30 nm)	—	10 fM	304

works, with dwell times in the range of milliseconds, but also at microseconds. In some cases, immunoassay and hybridization schemes use antibody-immobilised magnetic particles, that greatly improve the efficiency of the washing and separating steps of the captured antigens under a magnetic field.^{298,302}

Conclusions

The features of SP-ICP-MS for the detection, quantification and characterization of nanoparticles have led to the development of this technique and its increasing application in many different fields under analytical scenarios of varying complexity, which have been comprehensively covered in this review. However, its implementation in commercial instruments by most ICP-MS manufacturers can be considered the key to its success. Despite the rapid evolution of SP-ICP-MS in the last ten years, in 2020 the number of publications related to fundamental aspects of the technique and the development of methods was still 1 out of 3 with respect to the number of publications dedicated to specific analytical applications. For this reason, by following Horlick's approach,¹² although SP-ICP-MS is on the way of becoming a mature technique, its characterization stage cannot be considered finished yet. Moreover, its progress towards maturity is currently hampered by other issues like the availability of validated methods and the traceability of their results. Both issues are conditioned by the availability of reference materials, which are currently restricted to pristine nanoparticles of specific compositions, whereas matrix reference materials are still not available due long-term stability problems.¹⁹ This situation entails using nanoparticle suspensions supplied from a reduced number of manufacturers as standards for calibrations and quality control. Furthermore, since standards for only a small number of nanoparticle compositions are available, indirect calibrations based on the determination of the nebulisation efficiency and the use of

dissolved standards are applied routinely to obtain quantitative information. This approach requires that not only the element in dissolved and particulate forms behave in the ICP in the same way, but also the nebulisation for the standards and the samples. These two factors cannot be disregarded to obtain unbiased results, which is particularly challenging when dealing with complex matrices.

The analytical scenarios in which SP-ICP-MS is more frequently applied are those related to studies about fate and (eco)toxicity under controlled laboratory conditions involving the use of pristine nanoparticles (type 2 scenarios). The main reason is that both size and concentration of the nanoparticles added can be selected according to the experiment design and the detection capability, although there is an increasing trend to perform these studies at realistic concentrations. In any case, the most complex challenge for SP-ICP-MS is the analysis of samples originally containing nanoparticles, namely foods and environmental and biological samples (scenarios type 3), because of the complexity of the matrices and the low concentrations expected, but also nano-enhanced products (scenario 1). The main drawback with these samples is that the nature of the particles is unknown in many cases. Consequently, only masses of element per particle or equivalent sizes (assuming an expected composition and shape) can be reported, unless complementary techniques are used to obtain such information. An additional difficulty with this samples arises when part of the particle size distribution is missed due to the attainable size LODs. In such cases, nanoparticle concentrations will be underestimated and mean sizes overestimated. Hence, results should include these limitations, only reporting the occurrence of particles in the sample over a certain (equivalent) size and number concentration, considering SP-ICP-MS as a screening technique instead of a fully quantitative one. On the other hand, particles are not the only targets of SP-ICP-MS, its capability for the simultaneous quantification of dissolved elements has also

been exploited in many of the applications involving nanoparticles prone to oxidation or dissolution, like those made of Ag, CuO or ZnO.

Although SP-ICP-MS is mostly involved in methods for the analysis of samples containing nanoparticles, the technique is also suitable for being used in methods where nanoparticles are not analytes but analytical tools. This is the case of immunoassay and hybridization methods for the analysis of biomolecules, where nanoparticles are used as elemental labels conjugated to antibodies and oligonucleotides. By selecting the adequate nanoparticles, SP-ICP-MS can provide better limits of detection for these bioassays due to its high sensitivity in terms of number concentration. The applications of SP-ICP-MS in immuno- and hybridization assays are still scarce but show very promising results.

List of acronyms

AAS	Atomic absorption spectrometry
AES	Auger electron spectroscopy
AFM	Atomic force microscopy
AF4	Asymmetric flow field flow fractionation
ATR-FTIR	Attenuated total reflectance-Fourier transform infrared spectroscopy
AUC	Analytical ultracentrifugation
BSA	Bovine serum albumin
CE	Capillary electrophoresis
CFFF	Centrifugal field flow fractionation
CLS	Centrifugal liquid sedimentation
CNT	Carbon nanotube
CPE	Cloud point extraction
DE	Dissolved element
DF	Double focussing
DGT	Diffusive gradient in thin film
DLS	Dynamic light scattering
DMA	Differential mobility analysis
EDM-HSI	Enhanced darkfield microscopy-hyperspectral imaging
EDS	Energy dispersive X-ray spectroscopy
ETAAS	Electrothermal atomic absorption spectrometry
EXAFS	Extended X-ray absorption fine structure
FAAS	Flame atomic absorption spectrometry
FESEM	Field-emission scanning electron microscopy
FIBSEM	Focused ion beam scanning electron microscopy
FTIR	Fourier transform infrared spectroscopy
HAADF	High-angle annular dark-field
HDC	Hydrodynamic chromatography
HIM	Helium ion microscopy
HPLC	High performance liquid chromatography
HRTEM	High resolution transmission electron microscopy
IC	Ion chromatography
ICP-MS	Inductively coupled plasma-mass spectrometry
ICP-OES	Inductively coupled plasma-optical emission spectrometry
ISE	Ion selective electrode potentiometry
LA	Laser ablation
LDA	Laser diffraction analysis

LOD	Limit of detection
MALS	Multangle light scattering
NOM	Natural organic matter
NP	Nanoparticle
NTA	Nanoparticle tracking analysis
Q	Quadrupole
RI	Refractive index
SAM	Scanning Auger mapping
SDS	Sodium dodecylsulphate
SEC	Size exclusion chromatography
SEM	Scanning electron microscopy
SIMS	Secondary-ion mass spectrometry
SMPS	Scanning mobility particle sizer
SP-ICP-MS	Single particle-inductively coupled plasma-mass spectrometry
STEM	Scanning transmission electron microscopy
TEM	Transmission electron microscopy
TMAH	Tetramethyl ammonium hydroxide
TOF	Time of flight
TXRF	Total reflection X-ray fluorescence
UV-Vis	Ultraviolet visible absorption
XANES	X-ray absorption near edge structure
XAS	X-ray absorption spectroscopy
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction

Author contributions

Eduardo Bolea: writing – review & editing; Maria S. Jimenez: writing – original draft; Josefina Perez-Arantegui: writing – original draft; Juan C. Vidal: investigation, writing – original draft; Mariam Bakir: investigation, writing – original draft; Khaoula Ben-Jeddou: investigation, writing – original draft; Ana C. Gimenez-Ingalaturre: investigation, writing – original draft; David Ojeda: investigation, writing – original draft; Celia Trujillo: investigation, writing – original draft; Francisco Laborda: conceptualization, supervision, visualization, writing – review & editing.

Conflicts of interest

There are no conflicts to declare.

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CHAPTER 13

Single Particle Inductively Coupled Plasma Mass Spectrometry: A Valuable Tool in Environmental Analysis

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13.1 Introduction

Although colloidal and nano-size particles are naturally present in the environment, the growing development and use of nano-enhanced materials have led to recognising the relevance of the occurrence of engineered nanoparticles in different environmental compartments.^{1,2} In this regard, metrological limitations related to the detection, characterization and quantification of nanoparticles in environmental samples has been one of the main drawbacks and still remains a challenge.³ However, over the last ten years, the situation has been improving by the development of new analytical methods such as nanoparticle tracking analysis,⁴ field flow fractionation hyphenated with inductively coupled plasma mass spectrometry (FFF-ICP-MS),⁵ and especially single particle ICP-MS (SP-ICP-MS).⁶ The relevance of SP-ICP-MS

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The analytical demands in the environmental domain are not only constrained to the analysis of field samples (waters, soils, sediments, sludges, *etc.*). Moving from the field to the laboratory, tests under controlled conditions, involving pristine nanoparticle or products containing nanoparticles, are required for the risk assessment of nano-enhanced materials. These laboratory-scale tests involve the simulation of the release and transformation processes that nanomaterials may undergo during their whole life cycle, as well as ecotoxicological studies through *in vitro* and *in vivo* assays. In all these areas, SP-ICP-MS has proved to be a suitable technique for the analysis of complex samples, providing reliable information about engineered nanoparticles and their transformation products,¹² but also about naturally occurring or incidental nanomaterials.¹³

13.2 Basic Principles of SP-ICP-MS

The theoretical basis of single particle detection applied to ICP-MS was outlined by Degueldre and Favarger¹⁴ and summarized by Laborda *et al.*⁶ Basically, when a sufficiently diluted suspension (number concentrations of 10^8 L^{-1} or lower) is introduced into an ICP-MS, particles are present in just some aerosol droplets, arriving into the plasma randomly, whereas the dissolved species are homogeneously distributed in all the droplets. Measuring at very high data acquisition frequencies ($>100 \text{ Hz}$), with dwell times from milli- to microseconds, nanoparticles are detected as individual events over a continuous baseline due to the background or the dissolved element. The intensity of these events is related to the mass of the element in the nanoparticle, and hence to its size if additional information about the composition, shape and density of the nanoparticle is available, whereas the frequency of events is proportional to the number concentration of nanoparticles. Besides, information about dissolved forms of the element measured could also be obtained from the baseline.¹⁵

Depending on the dwell time selected, nanoparticle events can be recorded in two different forms. When using dwell times in the millisecond range (3–10 ms), larger than the duration of the ion cloud in the instrument, typically between 300 and 1000 μs , events are recorded as one-reading signals (pulses), whereas for dwell times in the microsecond range (10–200 μs), they are recorded as peaks (transient signals), comprising several readings (see Figure 13.1a). For pulses, the intensity of the event is given by the reading itself, whereas for peaks, the intensity is calculated as the sum of the individual readings along it.

Plotting the event intensity *vs.* the number of events from both milli- and microsecond time scan raw data, histograms with two distributions are obtained (see Figure 13.1b). The first distribution corresponds to the background and/or the presence of dissolved forms of the element measured and the second to the nanoparticles themselves. If the second distribution is

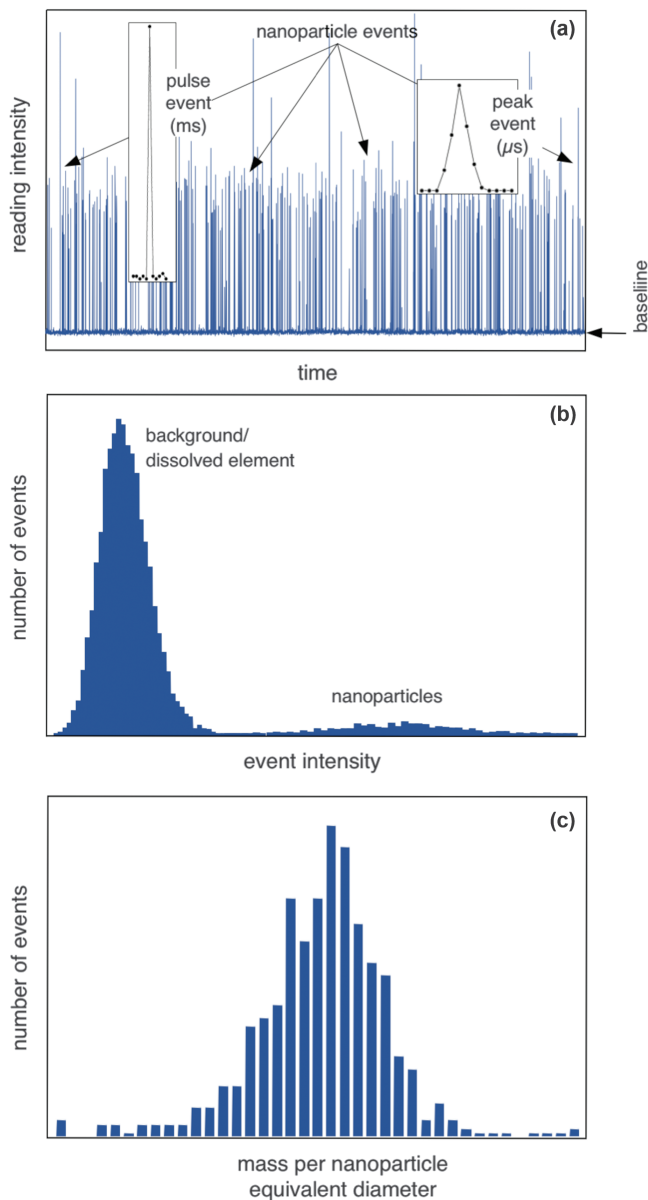


Figure 13.1 (a) Time scan of a suspension containing nanoparticles and dissolved forms of the same element. (b) Event intensity histogram of data from (a). (c) Mass per nanoparticle/size distribution of spherical nanoparticles calculated from the second intensity distribution in (b). Reproduced from ref. 12 with permission from the Royal Society of Chemistry.

further processed to convert the event intensities, the mass of the element per nanoparticle or size distributions can be obtained (see Figure 13.1c). Instrumental and metrological issues related to measurements, data acquisition and data processing have been summarized and described in detail in several reviews^{6–8,10} and articles therein.

The different types of quantitative information on nanoparticles achievable by SP-ICP-MS requires different calibrations, which must be obtained through the measurement of the corresponding standards. Whereas dissolved element mass concentration calibration involves conventional elemental standards prepared from dissolved species of the element of concern, particle number concentration and size calibrations require reference materials characterized in number concentration or size, respectively. In the case of number concentration, a gold nanoparticle standard has recently become available,¹⁶ although gold size standards with certified total metal concentration have also been used. Size or element mass per particle calibration should involve the use of size standards of nanoparticles with the same composition and shape as the nanoparticles under study. Because such standards are just available for certain nanoparticle compositions and not always in a wide range of sizes, indirect calibrations based on the use of dissolved standards of the element of interest, assuming that particles behave in the ICP-MS the same way as the dissolved element, and the determination of the nebulization efficiency are usually applied. Nebulization efficiency can be calculated by the frequency or the size methods,¹⁷ which requires nanoparticle size and dissolved element standards, most usually gold, but also by the dynamic mass flow, a reference material free method.¹⁸ An overview of calibration issues in SP-ICP-MS can be found in Laborda *et al.*¹⁹

13.3 Analytical Information and Figures of Merit

Two types of analytes are under the scope of SP-ICP-MS, namely nanoparticles and dissolved species. The information provided by SP-ICP-MS can be: (i) qualitative, about the presence of nanoparticulates and dissolved forms of specific elements; (ii) quantitative content, such as number and mass concentrations; and (iii) characterisation, about the mass of element/s per nanoparticle and nanoparticle size. Nevertheless, because the chemical composition of the particles is not usually available in many environmental samples, the size information is reported as an equivalent diameter, by assuming a specific composition (*e.g.*, metallic Ag, TiO₂) and a spherical shape.

In addition, other types of information can be obtained from SP-ICP-MS measurements, such as aggregation or agglomeration processes suffered by nanoparticles. This is especially relevant in environmental applications, like in the fate of nanoparticles in environmental conditions, since the constituents in these complex media (*e.g.*, organic matter) or the variations in pH or ionic strength, can cause changes in size, size distribution and concentration that can be monitored by SP-ICP-MS. Also, the dissolution processes of the nanoparticles can be studied since the technique allows the quantification of both

the particulate and the dissolved fractions of the element determined. All these topics will be discussed in detail in the following sections.

Figures of merit should be defined for both particle concentration and size (or mass of element per particle), but also for dissolved element mass concentration. Selectivity, precision, trueness, limits of detection (LOD) and measurement range have been regarded. Selectivity evaluation should consider not only polyatomic interferences, but also matrix constituents that may interfere with the nebulization even though large dilutions are often applied to prevent coincident particle events. Thus, changes in the nebulization efficiency may lead to bias on the particle number concentration, whereas changes in the ionization affect particle size. Precision has been evaluated through both repeatability and reproducibility, with repeatability values around 5% in size and 20% in number concentration, values that are even higher in the case of reproducibility. Higher bias is also commonly reported when considering number concentration with respect to particle size. The evaluation of trueness is limited to a recovery test due to the lack of certified reference material for nanoparticles in any type of matrix. In general, problems with discrimination of baseline signals from those of small particles and likely partial dissolution processes, which increase the baseline signal, are considered as the main contributions to the bias and low reproducibility reported.¹⁹ Also, the instability of the spiked standard in the samples may lead to low recoveries.²⁰ For all these reasons, the confirmation of the results by independent methods, such as electron microscopy, is highly recommended when possible.

A comprehensive list of size LODs for metallic and oxide nanoparticles can be found in the literature.¹¹ Best-case values around 10 nm were calculated for most common nanoparticles at 100 μ s dwell time using a commercial quadrupole instrument. These figures are improved when using double focussing spectrometers due to the better sensitivity attainable with these instruments, although the presence of dissolved forms of the element measured will degrade the LOD in either case. On the other hand, the number concentration LOD depends on the number of particle events detected in a blank and the acquisition time. As reference, the LODs of 100 particles per mL have been reported for acquisition times of 60 s.¹¹ Finally, the measurement range in number concentration depends on the occurrence of multiple particle events, which is proportional to the flux of particles through the plasma and the dwell time.²¹ With conventional nebulization systems, linear ranges up to 10^8 L⁻¹ can be achieved at microsecond dwell times. Linearity of size measurements are limited by low transport efficiencies and/or incomplete vaporization of large particles,^{22,23} together with the saturation of the detector.²⁴ For instance, linear size ranges up to 1 μ m for SiO₂ particles have been reported.²⁵

13.4 Environmental Applications of SP-ICP-MS

Over the last 10 years, SP-ICP-MS publications have evolved from fundamental studies, proofs-of-concept and development of methods to a wide

range of applications in a variety of fields and scenarios, including the environment.¹² In the environmental field, the most complex scenario corresponds to the analysis of environmental samples (waters, soils, sediments, wastes, sludges, organisms, *etc.*) because of the unknown nature of the nanoparticles present, the low concentrations expected and the complexity of most of the matrices involved. Laboratory studies for simulating the release and fate of nanoparticles or products containing nanoparticles under relevant environmental conditions, as well as ecotoxicological assays, are a second group of scenarios showing relatively less complexity, because they are performed under controlled conditions by using pristine nanoparticles of selected sizes and concentrations in most cases.

This section has been organized according to the three scenarios listed above: (i) environmental samples, (ii) release and fate laboratory studies involving nanoparticles or products containing nanoparticles and (iii) *in vitro* and *in vivo* ecotoxicological tests.

13.4.1 Analysis of Environmental Samples

The increasing use of consumer and industrial products containing engineered nanomaterials can lead to the release of nanoparticles during their whole life cycle, eventually ending up in the environment. Waters, but also soils and sediments, as well as the organisms living in them, are the main targets for monitorization, with special attention to wastewater treatment plants (WWTPs) due to their role in both the removal and the eventual discharge of nanoparticles to natural waters. In addition, the detection of incidental nanoparticles of different origins has also been a matter of concern.

13.4.1.1 Waters

Nano-enabled sunscreens containing TiO_2 are a source of such nanoparticles in recreational waters.^{26–30} For this reason, SP-ICP-MS has been used in several studies to determine TiO_2 nanoparticles in bathing areas. The analysis of water samples requires minimal sample preparation, which includes filtration or centrifugation to remove large particles. SP-ICP-MS results were complemented by transmission electron microscopy with energy dispersive X-ray spectroscopy (TEM-EDS)²⁹ and the detection of the organic component of sunscreens oxybenzone, confirming that sunscreen constituents, including Ti-containing nanoparticles, were present in the waters.^{28,30} On the other hand, differentiation between natural and anthropogenic nanoparticles was studied through the ratios Ti/Al and Ti/Fe .^{26–28}

TiO_2 nanoparticles are also used as additives in building materials. These materials can end up in landfills and potentially leach into the environment. Kaegi *et al.*³¹ analysed landfill leachates by SP-ICP-MS and other complementary techniques (dynamic light scattering, electron microscopy), detecting Ti-containing particles in the samples studied. Some of these

particles were very similar to the white pigments detected in facades, whilst others were associated to primary minerals.

Although the presence of nanoparticles in tap water could be considered negligible, corrosion by-products from the distribution pipes can be released as nanoparticles.^{32,33} The analysis of tap water by SP-ICP-MS confirmed the presence of Fe-, Pb-, Sn-, Ag- and Cu-containing nanoparticles. Fe and Cu particles were attributed to pipe corrosion, whereas Pb and Sn were associated with solder materials.³² In a different study, the presence of silver traces in the copper pipes was found to be responsible for the release of nanoparticulate Ag into tap water.³³

As mentioned above, WWTPs contribute to the removal of engineered nanoparticles, being important to evaluate the efficiency of the water treatment processes to control the discharge of nanoparticles into aquifers. The incidence of 13 types of nanoparticles throughout the wastewater treatment process was studied by SP-ICP-MS, analysing samples of influent, post-primary, effluent and reclaimed water.^{34,35} The efficient removal of nanoparticles was observed along the wastewater treatment, removing 84–99% of natural and engineered nanoparticles from the influent to the reclaimed water except for Mg, Ni and Cd, where the removal ranged from 70 to 78%. On the other hand, Li *et al.*³⁶ demonstrated that despite the effective removal of Ag-containing nanoparticles from wastewater influents, the effluents contributed to an increase in the concentration of such particles in the receiving rivers.

13.4.1.2 Sludges, Sediments and Wastes

Since the sludges from WWTPs will end up in landfill, incineration plants or applied to agricultural soils, the determination of the nanoparticle concentrations in these samples is also relevant. Due to the high ratio of dissolved *vs.* particulate elements, as well as the interactions of the nanoparticles with the matrix, sludge samples are processed in different ways to extract the metal-containing nanoparticles prior to SP-ICP-MS analysis. Tou *et al.*³⁷ used acetic acid and analysed the extracts by SP-ICP-MS. Results showed that billions of Ti-, Fe- and Zn-containing nanoparticles per gram of sludge were extracted, suggesting that these nanoparticles could be released into the environment under acidic environmental conditions. Ion exchange resins have been used for removing more than 50% of the dissolved fraction of the metals, allowing the detection of smaller nanoparticles.³⁴

A challenge in the analysis of environmental samples is the distinction between natural and engineered particles. Loosli *et al.*³⁸ analysed sewage sludge using a time-of-flight (TOF) instrument that allows multi-element detection and the determination of element ratios in individual particles. Their results revealed that natural TiO₂ particles were associated with elements like Al, Fe, Ce, Si, La, Zr, Nb, Ba, Th, Ta, W and U, with elemental ratios to Ti in agreement with average crustal ratios, whereas the presence of engineered TiO₂ particles was demonstrated and quantified by the Ti/Nb ratio.

In addition to the analysis of sludges, the impact of WWTPs can be monitored through the analysis of sediments close to WWTP discharge points. Metcalfe *et al.*³⁹ monitored Ag nanoparticles in sediments from a receiving lake, and determined whether silver could be released from such contaminated sediments. The results showed that most silver was associated with bottom and suspended sediments, although the element could be released both in the colloidal and dissolved fractions. These results demonstrated that contaminated sediments could be a continuing source of silver in the aquatic environment if sediments are re-suspended by currents, contributing to the exposure of aquatic organisms to silver.

Tou *et al.*⁴⁰ studied the occurrence and environmental implications of Ti- and Zn-containing nanoparticles in estuarine sediments, by applying different extraction methods and analysing the fractions extracted by SP-ICP-MS. Their results demonstrated that Ti-containing nanoparticles were mainly derived from the non-bioavailable fraction, which is considered safe according to conventional risk assessment methods. On the contrary, Zn-containing nanoparticles were mainly found in the bioavailable fraction, indicating a significant environmental risk from this element, which was the most abundant toxic metal found in the sediments.

As a consequence of mining activities, particles containing toxic elements can act as transport vectors of such elements, as it was demonstrated by Gomez-Gonzalez *et al.*⁴¹ by analysing mine tailing wastes containing arsenic by SP-ICP-MS in combination with synchrotron techniques. Their study confirmed that arsenic was released as colloidal scorodite, a poorly soluble iron(III) arsenate, which was originally considered a stable form of arsenic in the environment.

13.4.1.3 Microorganisms and Aquatic Organisms

Nanoparticles released in the environment can accumulate in biota, biomagnifying along the food chain. The trophic transfer of Ag- and Ti-containing nanoparticles was investigated in a lake catchment by analysing water, sediments and aquatic organisms (plankton, crustaceans, molluscs and fishes) by SP-ICP-MS.⁴² For the aquatic organisms, an alkaline digestion with tetramethylammonium hydroxide (TMAH) was used to extract nanoparticulate Ag and Ti. Results indicated that both types of particles were widely present in water and sediments, as well as in aquatic organisms, confirming the transfer of nanoparticulate Ag and Ti along the aquatic food chain. Similar studies demonstrated the presence of Ag^{43,44} and Ti-containing particles⁴⁵ in fishes and molluscs analysed by SP-ICP-MS.

The bioaccumulation of nanoparticles was also demonstrated by Xu *et al.*,⁴⁶ who analysed seawater and molluscs by SP-ICP-MS. Whereas seawater samples were analysed after dilution, molluscs were subjected to enzymatic digestion with pancreatin and lipase. Results indicated that particle concentration of Ti-, Cu-, Zn- and Ag-containing nanoparticles in molluscs were much higher than in the seawater, suggesting the bioaccumulation of the nanoparticles.

13.4.2 Laboratory Tests Involving Nanoparticles or Products Containing Nanoparticles

Whereas the analysis of environmental samples allows the occurrence of nanoparticles in the ecosystems to be known, as well as the exposure level of the organisms living in them, laboratory studies under relevant environmental conditions are used for simulating the release and fate of nanoparticles and their transformation products to understand their potential environmental risk.

13.4.2.1 Release Studies

The detection and characterization of the nanoparticles released from nano-enabled products under selected conditions is a common practice for predicting the environmental occurrence and exposure level to nanoparticles. Release studies based on the leaching of the nanoparticle-containing material with different types of waters are typically performed. Although ultrapure water has been used in some cases,^{47,48} natural and model waters (e.g., seawater,⁴⁹ freshwater,^{49,50} acidic rainwater⁴⁹) have also been used. More specific studies involved the use of stormwater runoff⁵¹ and rain and snow waters under weathering conditions.⁵² The studied materials included painted surfaces, in relation to the release of TiO_2 ⁵² and Cu_2O ⁵⁰ additives, TiO_2 and Ag enhanced textiles^{47,53} or Cu-treated woods.⁴⁸ Road dust has been studied in relation to the emission of Pt-containing particles from vehicle catalysts.⁵¹ Release studies by using SP-ICP-MS have not only been limited to inorganic particles, the technique has been used to confirm the release of incidental microplastics from consumer products by monitoring ^{13}C .⁵⁴ In these studies, sample preparation is limited to the separation by settling, filtration or centrifugation and the adequate dilution of the filtrate or the supernatant prior to SP-ICP-MS measurements for detection and size characterization of the particles released, as well as their quantification.

13.4.2.2 Fate Studies

In addition to the release of nanoparticles, the study of their stability and the transformations they undergo under environmental conditions (dissolution/oxidation, aggregation, *etc.*) is needed to evaluate their potential risk. SP-ICP-MS has proved to be an effective technique to evaluate the fate of nanoparticles in environmental conditions, because changes in size, size distribution and concentration can be monitored. In addition, the technique allows the quantification of both the particulate and the dissolved fractions of the element monitored, providing information about the dissolution of the nanoparticles.

A number of laboratory studies have been carried out to evaluate the changes that occurred when particles are brought into contact with natural waters,^{55–59} soils^{60–63} or sediments.^{63,64} Sludges and wastewaters have also been considered to study the fate of nanoparticles in WWTPs.^{65,66} Although

some studies considered the effects on Au,^{59,63,67} TiO₂^{59,61} or CeO₂,^{60,61} most of them involved metallic Ag^{56–59,64–67} or Ag-based nanoparticles (Ag₂S, AgCl).⁵⁵ These studies have shown that the behaviour of nanoparticles is influenced by their size, shape, chemical composition, coating and their initial concentration, as well as external conditions, such as temperature, pH or the presence of organic matter or chlorides.

Jimenez-Lamana *et al.*⁵⁶ studied the stability of Ag nanoparticles in lake water, showing that agglomeration depended on the surface coating, although natural organic matter improved their stability regardless of the coating. Wimmer *et al.*⁵⁵ confirmed that citrate coated Ag, AgCl and even Ag₂S nanoparticles were almost completely dissolved at environmental relevant concentrations in seawater within three days. They used SP-ICP-MS in combination with cloud point extraction for concentration and isolation of nanoparticles from dissolved silver species. The fate of Ag nanoparticles in wastewater was investigated by Azodi *et al.*,⁶⁶ confirming their dissolution followed by the subsequent formation of smaller Ag nanoparticles containing sulfur after 5 days. Donovan *et al.*⁵⁹ studied the effectiveness of conventional drinking water treatments to remove Au, Ag and TiO₂ nanoparticles from surface waters by using SP-ICP-MS. Their results indicated that lime softening removed most of the nanoparticles, whereas further treatment by alum coagulation combined with activated carbon adsorption resulted in complete removal of Au and Ag nanoparticles, and nearly complete removal of TiO₂ nanoparticles.

Soil studies involve the extraction of the nanoparticles prior to their detection by SP-ICP-MS. Apart from water,^{61,62} tetrasodium pyrophosphate (TSPP) has also been used as an extracting agent.^{60,64} Yang *et al.*⁶¹ demonstrated that after long term incubation of soils with ZnO, TiO₂, CeO₂ and Ag nanoparticles, less than 1% of the TiO₂, CeO₂ and Ag nanoparticles were released into water, whereas zinc was only released in dissolved forms. Torrent *et al.*⁶² also studied the adsorption and desorption of Ag nanoparticles in soils, confirming the high retention of Ag nanoparticles in the soils studied, as well as the release of dissolved silver. In the case of CeO₂ nanoparticles, Liu *et al.*⁶⁰ observed no significant change in particle size and concentration in soils aged for one month. The results obtained by El Hadri *et al.*,⁶³ using Au nanoparticles as model nanomaterial, suggests that the transport of engineered nanoparticles in soils and sediments is limited due to their hetero-aggregation with soil colloids.

13.4.3 *In Vitro* and *In Vivo* Ecotoxicological Tests

The bioaccumulation and toxicity of nanoparticles are processes still poorly understood, making *in vitro* and *in vivo* ecotoxicological tests worth studying in order to assess the behaviour and effects of engineered nanoparticles on living organisms. The use of SP-ICP-MS in this context is hampered by the complexity of the biological matrices involved and the need to extract the nanoparticles and their transformation products by using reliable digestion methods.

13.4.3.1 In Vitro Studies

In relation to the risk assessment of nanomaterials, different species of bacteria and algae have been investigated to study the uptake, biotransformation and ecotoxicity of nanoparticles under *in vitro* controlled conditions.

Mixtures of TiO₂, ZnO and Ag nanoparticles were added to artificial treated wastewater mixed with river water to evaluate the effect of nanoparticles on the river bacterial community.⁶⁸ After exposure, water samples were analysed by SP-ICP-MS to quantify the concentration of nanoparticles and dissolved species and to determine the mean size and size distributions of nanoparticles. Gomez-Gomez *et al.*⁶⁹ studied the uptake and biotransformation of Te nanoparticles by strains of *Staphylococcus aureus* and *Escherichia coli*. A biotransformation from nanospheres to nanorods was confirmed by TEM and X-ray diffraction (XRD), whereas SP-ICP-MS was used after lysis of the bacteria to determine the number of particles per bacteria and to estimate the dimensions of the rod-shaped Te nanoparticles, confirming the accumulation of nanoparticles in the microorganisms.

In addition to the bacterial strains, algae have also been used for *in vitro* ecotoxicity tests. The model green algae *Chlamydomonas reinhardtii* was studied in a wastewater matrix to assess its interaction with Ag nanoparticles.⁷⁰ SP-ICP-MS was used to differentiate the dissolved fraction from nanoparticles, to quantify the bio-uptake of both silver species and to monitor the aggregation and dissolution of the nanoparticles. Dissolved silver bioavailability was decreased because of the water matrix and the presence of ligands, nonetheless Ag nanoparticles were more bioavailable to algae.

The ecotoxicity of chromium to the algae *Raphidocelis subcapitata* was investigated by the addition of Cr(III) and Cr(VI) to the International Organization for Standardization (ISO) medium for algal ecotoxicity testing.⁷¹ SP-ICP-MS enabled the detection of incidental Cr-bearing nanoparticles after the addition of soluble Cr(III), linking the ecotoxicity to the presence of nanoparticles and/or ionic chromium. SP-ICP-MS was also used for the size determination of the biogenic nanoparticles produced by the algae *Aureobasidium pullulans*, which was incubated for 30 days in a culture media containing selenium and tellurium.⁷²

13.4.3.2 In Vivo Studies

The assessment of nanoparticle ecotoxicity in terrestrial environments has been conducted by studying their uptake, bioaccumulation and impact on model soil organisms. These studies involved the spiking of soils and sediments with pristine nanoparticles, such as Ag,^{73–75} Au^{73,76} and CuO.^{77,78} Model soil organisms included earthworms like *Lumbriculus variegatus*,^{73,74} *Lumbricus rubellus*,⁷⁵ *Caenorhabditis elegans*⁷⁶ and *Eisenia andrei*⁷⁸ and the springtail *Folsomia candida*.^{77,78} Apart from water extraction,⁷⁴ as earthworms are complex biological matrices, enzymatic⁷⁵ and alkaline^{73,76} digestions were used prior to SP-ICP-MS analysis.

Size and coating had a minimal effect on the bioaccumulation of Ag nanoparticles in earthworms when added to sediments, whereas small

nanoparticles were more bioavailable from waters.⁷⁴ SP-ICP-MS served for quantification of the body burden, but also for the speciation of dissolved/nanoparticulate Ag accumulated in the earthworm tissue, being mainly present as dissolved forms.⁷⁵ SP-ICP-MS was also used for the quantification of Au nanoparticle uptake by *Caenorhabditis elegans* and the study of their biotransformation and aggregation in the gut.⁷⁶ Soil texture and nanoparticle concentration were reported to induce toxicity of CuO nanoparticles on *Folsomia candida*,⁷⁷ affecting their reproduction and growth at low concentrations in a loamy soil. Whereas in this study the toxicity was attributed to the particulate forms of CuO, in the work by Velicogna *et al.*,⁷⁸ the toxicity was related to the dissolution of the nanoparticles.

Ecotoxicological assays with Ag, Au, CeO₂ and TiO₂ in aquatic environments have been investigated using a variety of fish species, such as zebra fish (*Danio rerio*),^{79,80} trout (*Oncorhynchus mykiss*),^{81,82} and northern pike (*Esox lucius*),⁵⁷ as well as other aquatic model organisms, like the planktonic crustacean *Daphnia magna*,^{73,83} the amphipod *Hyaella azteca*^{84,85} and the mussel *Mytilus galloprovincialis*.⁸⁶ The uptake of nanoparticles by these organisms was performed by SP-ICP-MS after enzymatic or alkaline digestion tissues, although the hemolymph of *Daphnia magna* was analysed directly.⁸³ The *in vivo* formation of TiO₂ nanoparticles in mussels after titanium exposure was also confirmed by SP-ICP-MS.⁸⁶

The development of nanofertilisers for agriculture and the environmental exposure of plants to engineered nanoparticles has led to several *in vivo* studies with edible plants (tomato, pumpkin, soybean, cucumber, cress, mustard, lettuce, kale, wheat, rice, radish). These studies mainly consisted of the germination and growth of the plant seeds in a growing medium (hydroponic solutions^{87–96} or soils^{97–102}) spiked with different types and concentrations of nanoparticles (Au,^{88,90,97} Ag,^{88,89,91,99,101} Ag₂S,⁸⁷ Cu,¹⁰¹ CeO₂,^{92,98,102} CuO/Cu(OH)₂,^{92,103,104} TiO₂,^{92–94} Pd,⁹⁵ Pt,¹⁰⁰ ZnO^{96,101}). Nanoparticle uptake by roots and translocation to leaves was evaluated by the analysis of the roots, leaves and fruits of the plants.^{87,91,94,100} In the case of Ag nanoparticles, their uptake was affected by the size and concentration of the nanoparticles, with small-sized nanoparticles more easily absorbed.⁸⁹ The detection by SP-ICP-MS of biogenic nanoparticles in leaves after exposure to ionic silver has also been reported.⁹¹ Foliar exposure to nanoparticles has also been studied for Ag nanoparticles,⁹¹ whose transformation to ionic silver was reported, and CuO nanoparticles, which were retained on the leaf surface.^{103,104} However, Wang *et al.*⁸⁷ reported the uptake of Ag₂S nanoparticles by edible plants and their translocation to leaves without transformation or dissolution.

Analysis of plant tissues by SP-ICP-MS involves in most cases their enzymatic digestion with macerozyme R-10 to break down vegetal cells while preserving nanoparticle characteristics,^{87–91,93–101,104} although other extraction procedures have been proposed, as natural deep eutectic solvents for CeO₂, TiO₂ and CuO extraction from radish⁹² or the use of methanol for the extraction of copper-based nanoparticles from lettuce leaves to avoid their dissolution.¹⁰³

13.5 Benefits and Limitations of SP-ICP-MS in Environmental Analysis

In spite of the features of SP-ICP-MS, solving analytical problems involving nanoparticles in environmental samples is a complex task that requires the combination of different analytical techniques in most cases. The simplest approach is based on the use of ICP-MS, both in standard and single particle modes, in conjunction with an electron microscopy technique (e.g., TEM-EDS). Standard ICP-MS allows the determination of the total content of the element, whereas TEM-EDS allows the visualization of the particles, providing information about the morphology and composition if their concentration is not too low. In most cases, the nature of the nanoparticles detected by SP-ICP-MS is unknown, being described as element-containing particles, characterised by their element mass content per particle or an equivalent diameter by assuming a specific composition.

On the other hand, when the samples contain nanoparticles below the attainable size (or element mass per particle) LOD, the nanoparticle population will be partially recorded and the average size information will be overestimated, whereas the number concentrations will be underestimated. Under such constraints, the size and concentration information reported cannot be considered quantitative and should be restricted to confirm the presence of nanoparticles containing the element measured over a certain equivalent diameter and number concentration.

Although the implementation of SP-ICP-MS in commercial instruments by most ICP-MS manufacturers can be considered the key to the success of the technique, SP-ICP-MS is still on its way to become a mature technique, hindered by issues like the availability of validated methods and the traceability of their results, both of them conditioned by the availability of reference materials. In any case, the number of users and environmental applications is steadily increasing, mainly because the nano-environmental community is aware that SP-ICP-MS methods are both selective and sensitive enough to analyse inorganic nanomaterials in complex environmental samples.

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Comparative study of extraction methods of silver species from faeces of animals fed with silver-based nanomaterials

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Abstract

Extractions methods based on ultrapure water, tetramethylammonium hydroxide (TMAH), and tetrasodium pyrophosphate (TSPP) were applied to faeces collected from two in vivo experiments of pigs and chickens fed with a silver-based nanomaterial to study the fate and speciation of silver. For TMAH extraction, cysteine and CaCl_2 were used to evaluate their stabilization effect on the silver forms. The analytical techniques single-particle inductively coupled plasma mass spectrometry (SP-ICP-MS), hydrodynamic chromatography hyphenated to ICP-MS (HDC-ICP-MS) and asymmetric flow field flow fractionation coupled to ICP-MS (AF4-ICP-MS) were applied to the simultaneous detection of particulate and dissolved silver. Results have shown that water extraction was a suitable option to assess the environmental release of silver, with percentages of 3 and 9% for faeces of pigs and chickens, respectively. The use of TMAH extraction combined with SP-ICP-MS analysis was useful to characterize Ag-containing particles (less than 1%). Both stabilizers, cysteine and CaCl_2 , have a similar effect on silver nanoparticle preservation for chicken faeces, whereas cysteine-Triton was better for pig samples. In any case, silver extraction efficiency with TMAH was low (39–42%) for both types of faeces due to a matrix effect. TSPP followed by ICP-MS enabled the fractionation of the silver in the faeces, with silver sulphide (41%) and ionic silver (62%) being the most abundant fractions.

Keywords Silver fractionation · Nanomaterials · Liquid extraction · Single-particle ICP-MS · Hydrodynamic chromatography

Introduction

The increase in the use of engineered nanomaterials (ENMs) and their release into the environment makes necessary the evaluation of their potential risks. Silver nanoparticles (AgNPs) are widely used for their antibacterial properties [1]. They are incorporated in several products like medical and cosmetics products, food additives, textile coatings or food packaging [2, 3]. AgNPs are also studied as feed additives for animals [4, 5] with the aim of substitution of antibiotics. The elimination of the consumed AgNPs through the faeces can be one of the sources of the release into the environment. Information about the silver forms in the excreta of animals fed with nano-additives is part of the

recommended risk assessment of nanomaterials in the guidance of the European Food Safety Authority [6]. The toxicity of AgNPs depends on the particle properties (size, form, concentration) [7]. Subsequently, a suitable method for NPs characterization is essential for the risk assessment of nanomaterials, but, until the moment, there is no standard method for the characterization and quantification of NPs in faeces due to the complexity of the nature of excrements making the determination of silver species challenging.

Several sample preparation methods have been studied to digest biological samples containing NPs [8]. Acid digestion is frequently used to digest the nanoparticle-containing tissues [9, 10]. However, the strong acids dissolve the nanoparticles; thus, other extraction methods that enable the conservation of the integrity of nanoparticles are needed.

For most of the biological samples, alkaline extraction with TMAH [11–16] and enzyme-based extraction [17–21] are commonly used. TMAH is a strong base able to break organic and biological matrices [22]. To avoid changes in the NP morphology, a stabilizer is needed. Clark et al. [23] demonstrated

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that the use of TMAH alone caused the precipitation of Ag(I) in spiked fish, which was also demonstrated by Vidmar et al. [18] in a human placenta. Therefore, CaCl_2 was proposed as a stabilizer as it avoided Ag(I) precipitation. Jiménez-Lamana et al. [15] used cysteine to stabilize Ag(I) and Triton X-100 as a surfactant to stabilize AgNPs. The use of surfactants was reported in other studies [22, 24, 25] to prevent the loss of NPs and to recover the total spiked Ag. Gao et al. [24] also studied the process of sample grinding and concluded that it improved NP extraction leading to recoveries of nearly 100% for gold nanoparticles in mice organs. Enzymatic extraction is used for its ability to extract NPs without alterations from the tissues [21]. Generally, enzymatic extraction is conducted using proteinase K [26], but this enzyme can partially dissolve AgNPs, and the presence of matrix residues leads to a lower nanoparticle recovery [17]. Comparisons between alkaline and enzymatic extractions were reported [25, 27], obtaining better results with the alkaline extraction. Sung et al. [27] observed that TMAH gave higher recoveries (102%) than enzymatic extraction (74%) for the extraction of AgNP and AuNP from zebrafish liver samples.

Tetrasodium pyrophosphate (TSPP) has been frequently used for the extraction and fractionation of the different element species with special attention to particulate forms [28–30]. Hong et al. [30] developed a sequential extraction for different species of Ag (Ag(I), AgNPs, AgCl and Ag_2S) in spiked soils based on TSPP and sodium sulphide (Na_2S) measuring the resultant solutions by inductively coupled plasma optical emission spectrometry (ICP-OES). This method provided useful information about the nature of the Ag species in the spiked samples and their proportion. However, the use of aqueous ammonium and Na_2S as extractants caused a dissolution and complexation of Ag_2S and AgCl, leading to a loss of information about the size and morphology of the particles.

Water extraction is used to study the fate and transformations (e.g., dissolution, oxidation, aggregation) of NPs during the release process [31]. Animal manure is used as organic amendment to improve soil fertility [32]. However, these amendments can be a sink for metal accumulation, being important to evaluate their mobilization into the environment using water extraction protocols [33]. Water extraction followed by AF4 analysis was used for the size characterization of nanocolloids, macromolecules and microparticles in compost [34]. The authors observed different mobilization behaviours of several metals depending on their affinity of association for the particulate or macromolecule fractions. Water extraction of soils containing faeces of earthworms fed with cobalt NPs, AgNPs and Ag (I) revealed a silver concentration below the detection limit [35].

The objective of this study is to evaluate the efficiency of three different strategies, based on the use of water, TMAH and TSPP, for the extraction of dissolved and nanoparticulate silver species from faeces of pigs and chickens that were

orally fed with a silver-based nanomaterial. The behaviour of the silver species along the three extractions protocols will be studied by using different ICP-MS-based techniques (SP-ICP-MS, HDC-ICP-MS and AF4-ICP-MS).

Materials and methods

Chemicals

Aqueous gold solutions were prepared from a standard stock solution of $999 \pm 4 \text{ mg L}^{-1}$ (Panreac, Barcelona, Spain) by dilution in ultrapure water (Milli-Q Advantage, Molsheim, France) by accurately weighing ($\pm 0.1 \text{ mg}$). Aqueous silver (I) (Ag(I)) solutions were prepared from a standard stock solution of $994 \pm 3 \text{ mg L}^{-1}$ (Panreac, Barcelona, Spain) by dilution in ultrapure water. Diluted suspensions of AgNPs were prepared from commercial suspensions of AgNPs with sizes 10.3 ± 2.1 , 19.1 ± 3.6 , 39 ± 5 and $59 \pm 6 \text{ nm}$ (Nanocomposix, San Diego, USA). AuNPs of $47.8 \pm 1.8 \text{ nm}$ (Nanocomposix, San Diego, USA) with a concentration of 52 mg L^{-1} were used as a certificated material of size and concentration.

For the mobile phase used in HDC separation, a solution of 0.45 mM sodium dodecyl sulphate (SDS) (Bio-Rad Laboratories, California, USA, electrophoresis purity reagent, $\geq 98.5\%$) and 1 mM D-Penicillamine (PA) (Sigma Aldrich, St. Louis, USA, 98–101%) in 1 L of ultrapure water was prepared. To reach the pH 7.5 necessary for the measurements, a sodium hydroxide solution (Prolabo, Fontenay-Sous-Bois, France, 98%) and a nitric acid solution (J.T. Baker, Phillipsburg, USA, 69/70%) were used.

The extracting reagents used were TMAH 25% (Alfa Aesar, Kandel Germany, electronic grade, 99.9999 %), TSPP (Sigma-Aldrich, Sta. Louis, USA, $\geq 95\%$) and Na_2S (Sigma-Aldrich, Sta. Louis, USA, $\geq 99.99\%$ trace metals basis). Cysteine (Sigma-Aldrich, Sta. Louis, USA, BioUltra, $\geq 99.0\%$), Triton X-100 (Sigma-Aldrich, Sta. Louis, USA, laboratory grade), CaCl_2 (Fisher Scientific, Loughborough, UK, analytical reagent grade) and aqueous ammonium (NH_3) (30%, J.T.Baker, Phillipsburg, USA, $\geq 99.5\%$ trace metals analysis) were also used.

Instrumentation

ICP-MS in conventional mode and SP-ICP-MS analysis were conducted using a Perkin Elmer model NexION 2000 ICP mass spectrometer (Toronto, Canada). The sample introduction system consisted of a baffled cyclonic spray chamber and a concentric nebulizer (Meinhard, Colorado, USA). The isotopes monitored were ^{107}Ag and ^{109}Ag . Syngistix™ 2.5 (Perkin Elmer, Toronto, Canada) software, and the nano module for the SP-ICP-MS measurements were used for

the acquisition of the data. The experimental conditions are described in table T1 in supporting info (SI). Before all the analysis, ICP-MS measurement conditions were optimized according to the manufacturer instructions, and to achieve the maximum sensitivity for Ag, nebulizer gas flow and lens voltage were optimized using a $1 \mu\text{g L}^{-1}$ Ag standard. The transport efficiency, required for SP-ICP-MS calculations, was determined by the size method developed by Pace et al. [36] which relies on a calibration with ionic Au standards and the use of a Au NP size standard (50 nm).

For HDC-ICP-MS analysis, the high-performance chromatographic system used was a Waters 2796 Bioseparations module (Waters Corporation, Milford, USA). HDC separations were performed with a PL-PSDA Type 1 column (Agilent Technologies, Germany) with a nominal separation range of 5–300 nm, a length of 80 cm and an internal diameter of 7.5 mm. The sample flow rate used was 1.6 mL min^{-1} , and the mobile phase used was 0.45 mM of SDS and 1 mM of PA, optimized in previous studies [37]. It was coupled to a Perkin Elmer Sciex model ELAN DRC-e ICP mass spectrometer (Toronto, Canada) and used as a detector. The outflow of the column was delivered directly to the nebulizer of the spectrometer which is a glass concentric slurry nebulizer with a cyclonic spray chamber (Glass Expansion, Melbourne, Australia). Before the analysis, ICP-MS measurement conditions were optimized to achieve the maximum sensitivity for Ag, using a $10 \mu\text{g L}^{-1}$ Ag (I) solution. The experimental conditions of the HDC and the ICP-MS are shown in table T1 in SI. The quantification of the peaks of the chromatograms is reported by Jiménez et al. [37].

For AF4-ICP-MS analysis, an AF2000 (Postnova Analytics, Landsberg, Germany) system was used. The trapezoidal channel was 14 cm in length and 2 cm in width, and the spacer used was 350 μm thick (Postnova Analytics, Landsberg, Germany). As an accumulation wall, an ultrafiltration membrane of polyether sulfone (PES) (cut-off 5 kDa; Postnova Analytics, Landsberg, Germany) was utilized. The mobile phase was ultrapure water, previously filtered. The crossflow programs are listed in table T2 in SI. The AF4 system was coupled to a Perkin Elmer Sciex model ELAN DRC-e ICP mass spectrometer, used as a detector. The outflow of the channel was delivered directly to the nebulizer of the spectrometer which is a glass concentric slurry nebulizer with a cyclonic spray chamber. Before the analysis, ICP-MS measurement conditions were optimized to achieve the maximum sensitivity for Ag, using a solution with a concentration of $20 \mu\text{g L}^{-1}$. The experimental conditions of the AF4 and the ICP-MS are shown in table T1 in SI.

For transmission electron microscopy (TEM) analysis, a Tecnai F30 (FEI Company, Eindhoven, Netherlands) equipped with an electron source with a working voltage between 200 and 300 kV was used. It was coupled to an energy-dispersive spectrometer (EDS). Twenty microliters

of the sample digested (without no cleaning step prior the measurement) was deposited on a copper grid for the water extraction and on a gold grid for the alkaline extraction, and coated with carbon, once the sample was dry. To process the images obtained, ImageJ 1.53 version software was utilized.

Origin 9.6.5.169 (OriginLab, Northampton, MA, USA) was used for the processing of the data.

Samples

Pig and chicken faeces were collected from an in vivo experiment in which the animals were fed with feed containing the silver-kaolin. Before sample treatment, lyophilized faeces were ground with a Restch MM400 mill (Restch, Düsseldorf, Germany). The grinding was done in stainless steel jars with a 25-mm ball at a frequency of 25 Hz for 3 min. The total Ag content was obtained by acid digestion and ICP-MS determination. The detailed results will be published. Mean total concentrations of silver in faeces obtained were 155 ± 30 and $1026 \pm 178 \text{ mg kg}^{-1}$ for pigs fed with silver-kaolin at a silver concentration of 20 and 200 mg kg^{-1} , respectively, and an amount of Ag of $75 \pm 12 \text{ mg kg}^{-1}$ in faeces of chickens fed with 20 mg kg^{-1} of silver.

Procedures

Water extraction

One hundred milligrams of ground faeces was weighed ($\pm 0.1 \text{ mg}$) and added to 1 mL of ultrapure water, to obtain a solid/reagent ratio of 1:10 as described by Kosson et al. [38]. The suspensions were shaken at 28 rpm for 48 h at room temperature and darkness. Afterwards, the suspensions were sonicated for 5 min and left for 20 min at room temperature. Then, the samples were centrifugated at 7000 g for 5 min at 21°C to deposit the particles larger than $1 \mu\text{m}$ in the bottom of the tube.

Alkaline extraction

For the alkaline extraction, the protocol proposed by Jiménez-Lamana et al. [15] was used. One hundred milligrams of ground faeces was put into 15-mL polypropylene tubes with 2 mL of TMAH 25% (w/w) and 0.4 mL of cysteine 0.5% (w/w). They were mixed in a tumbler for 24 h at 28 rpm at room temperature and in darkness. After that, the solutions were sonicated and made up to 10 mL with a solution of cysteine 0.1% and Triton X-100 0.05%. Two different separation modes were studied to investigate the loss of Ag due to the centrifugation process. After the alkaline extraction, the centrifugation (at 7000 g for 17 min at 21°C) and the sedimentation were compared as separation methods. The sedimentation was done leaving the samples for 1 h in an ice

bath to the deposition of particles larger than 1 μm in the bottom. Samples were diluted prior to their analysis.

TSPP sequential extraction

For the sequential extraction process, the protocol developed by Hong et al. [30] was employed. Five hundred milligrams of ground faeces was added to 8 mL of 10 mM TSPP, and the pH was adjusted with a solution of 2 mM of NaOH. Samples were shaken at 200 rpm and left in an ultrasound bath for 30 min. The samples were centrifugated for 10 min at 157 g. The supernatant was separated from the solid. The same procedure was carried out on this pellet again: 8 mL of the solution of TSPP, agitation, ultrasonication and centrifugation. Once it was centrifuged, the solid was separated from the supernatant, and the latter was combined with the first supernatant that had been extracted. The same procedure was performed with the remaining pellet, but, in this third case, only 4 mL of 10 mM TSPP was added. In total, the resulting supernatant was around 18 mL. This supernatant was divided into 3 subsamples: direct determination through ICP-MS representing the Ag(I), AgNPs and AgCl-NPs; centrifugation and determination by ICP-MS, representing the Ag(I) and adding NH_3 , centrifugation and determination by ICP-MS representing the Ag(I) and AgCl-NPs.

The resulting pellet was washed 3 times with ultrapure water, and 8 mL of 0.1 M of Na_2S was added. The procedure that was followed is the same as that followed with the TSPP, and the suspension was shaken, ultrasonicated and centrifuged. The process was repeated two more times (with 8 mL and 4 mL more of Na_2S), obtaining approximately 18 mL of supernatant. With this step of fractionation, the Ag_2S -NP fraction was extracted.

Results and discussion

Study of the different extraction methods for the extraction of Ag species from faeces

Ultrapure water, alkaline extraction with TMAH and sequential extraction with TSPP and Na_2S were evaluated as extraction reagents of silver forms from faeces of pigs and chickens fed with feed containing a silver-kaolin nanomaterial.

Water extraction

Water extraction is considered an approach that evaluates the potential release of substances from waste materials [38]. Figure 1 shows the percentage of total Ag released in each extraction versus the total Ag obtained by the acid digestion, whose mean values are in the samples section. The total Ag released in faeces leachates using ultrapure

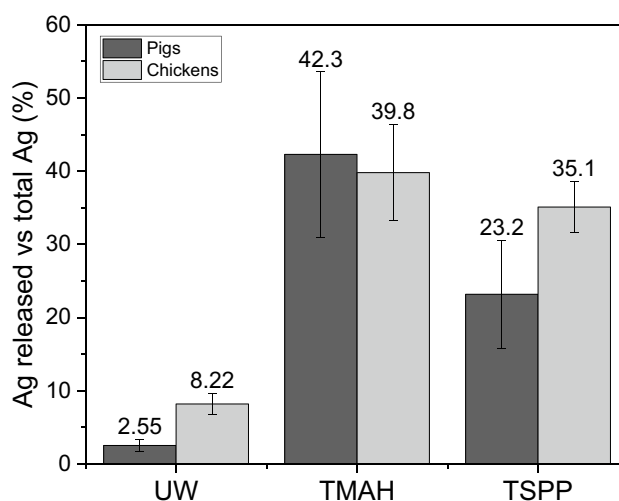


Fig. 1 Percentage of total extracted silver from faeces of pigs and chickens using ultrapure water, TMAH with cysteine and Triton-X and TSPP sequential extraction by ICP-MS

water, measured by ICP-MS, was $2.5 \pm 0.9\%$ for pigs and $8.2 \pm 1.4\%$ for chickens. This little percentage of release means that the impact of the faeces in the environment would be low if they were used as fertilizers. The different silver species in the leachates were characterized by different techniques: SP-ICP-MS, HDC-ICP-MS and AF4-ICP-MS. Concerning SP-ICP-MS analysis (Fig. 2), few silver particles were found; between 0.1 and 10% of the total Ag was released in a nanoparticulate form. The detection of the NPs in SP-ICP-MS analysis is limited to the size limit of detection (LOD_{size}) [39], which depends on the dissolved species concentration in every sample. When the concentration of the dissolved species increases, so do the baseline and the LOD_{size} . The smallest particles will not be detected, and they were included in the baseline; thus, the particle concentration is underestimated [40]. In our samples, because of the high concentration of dissolved silver, not all the particle distribution is seen, and only the tail of the distribution is obtained. In general, the LOD_{size} obtained for the samples was around 35–40 nm, which means that particles with a smallest size could not be quantified as particles but as dissolved form, and their concentration is underquantified. The most frequent mass of silver per particle found was less than 1 fg per particle (0.781 fg for chicken and 0.971 fg for pigs), which is equivalent to a diameter of 34 ± 2 nm for chickens and 35 ± 9 nm for pigs (figure S1 in SI).

In Fig. 3, the HDC-ICP-MS chromatograms obtained for the water extraction of pig (Fig. 3a) and for chicken (Fig. 3b) faeces are shown. Two peaks were seen in both chromatograms, one peak eluted at 8.96 ± 0.04 min, which corresponds to Ag(I) (chromatogram of standard of Ag(I)

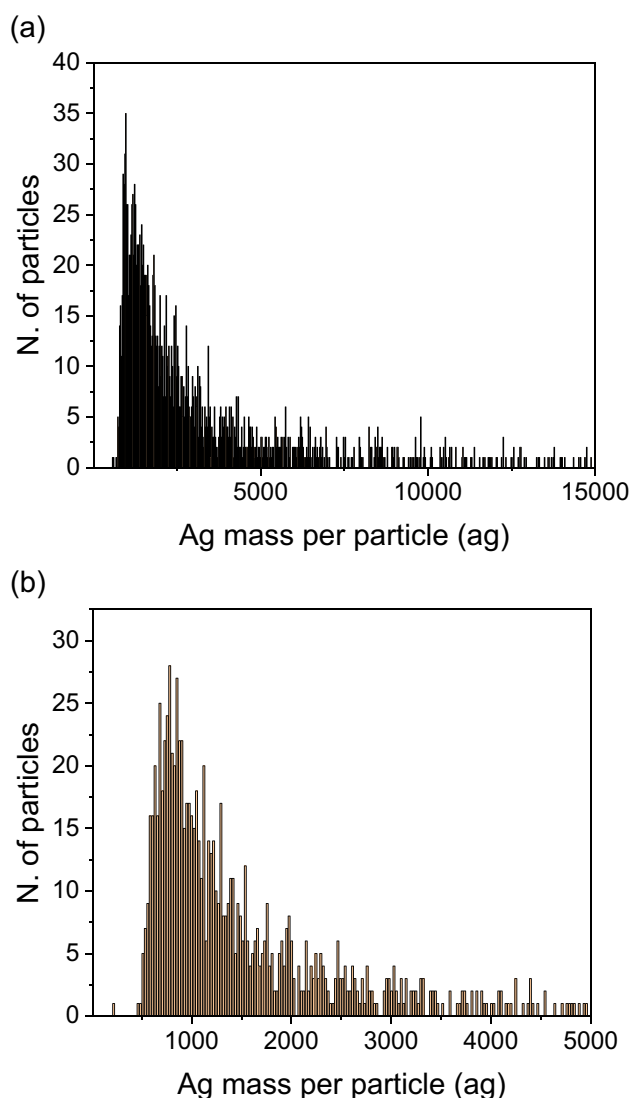


Fig. 2 Distribution of Ag-containing particles in the leachates of (a) pigs and (b) chickens faeces by SP-ICP-MS

in water is shown in figure S2 in SI) and appears in both animal leachates. For pigs, another peak was observed at 7.73 min corresponding to the elution of Ag-containing particles with sizes larger than 300 nm, which is the size limit of the HDC column that the manufacturer set as the maximum size of separation. For chicken faeces, a second peak appears at 8.52 min in the leachates, which could correspond to another form of Ag(I) complexed to other biomolecules. These findings agree with TEM results (figure S3 in SI), in which particles and agglomerates with sizes between 100 nm and 1 μ m were found. With EDS, the composition of some of these complexes included Ag_2S , while Al and Si observed in the EDS spectrum confirmed the presence of other particles that were bound to kaolin which is the support used in the nanomaterial fed to the animals [41].

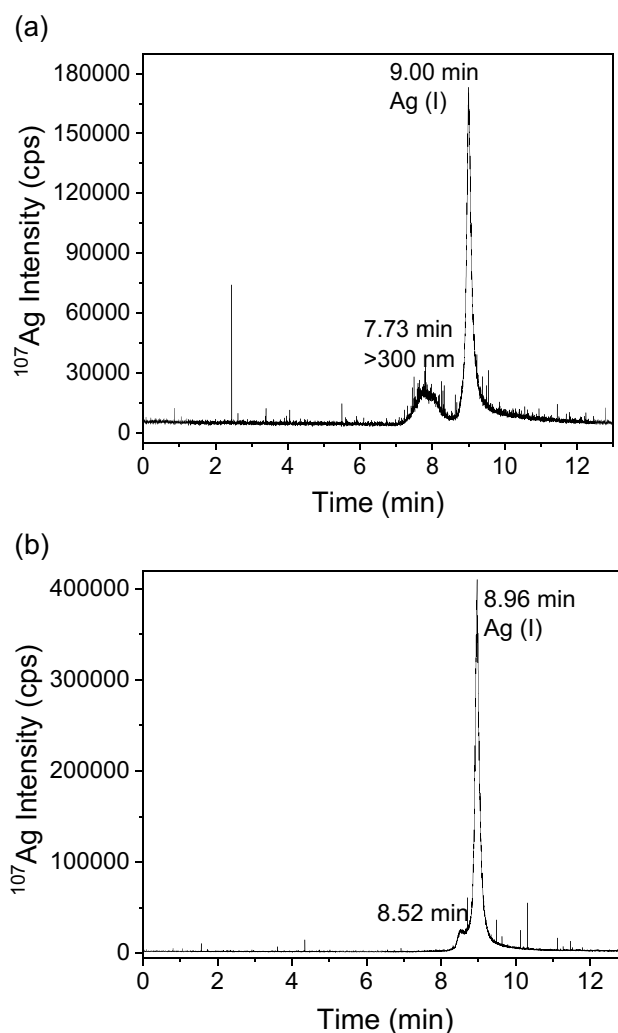


Fig. 3 HDC-ICP-MS chromatograms for the aqueous leachates of (a) pigs and (b) chicken faeces

The leachates of pig faeces were also measured by AF4-ICP-MS. The results obtained agree with the ones obtained by HDC-ICP-MS (Fig. 3a) and TEM (figure S3 in SI). In the fractogram obtained (figure S4a in SI), there is a small peak at 6.40 min corresponding to dissolved silver forms and another big peak which corresponds to bigger particles. Several dilutions of a sample were analysed to verify the unalteration of the particles during the process. The comparison of the fractograms resulting from the different dilutions shows that depending on the dilution, the particle retention time changes, shifting into shorter retention times as the dilutions increased (figure S4b in SI). This could mean that the particles size changes depending on the dilutions due to changes in the equilibriums inside the sample. Therefore, water is not a good extractant of the particles in this matrix, due to its inability to maintain the size of the particles and its dependence on the dilutions.

Alkaline extraction

TMAH extraction was used in the bibliography to extract AgNPs from several biological tissues [11, 12, 22, 24, 26, 42]. TMAH is capable to digest organic and biological matrices due to its ability to break down the disulphide chemical bonds of proteins [12, 22] without changes in the Ag(I) and AgNPs. In the study of Jiménez-Lamana et al. [15], cysteine was added to the solution to stabilize Ag(I) by binding it because of its thiol groups [43, 44]. Triton X-100 is a non-ionic surfactant, and it is added to the extraction solutions to stabilize the small particles due to lower surface tension [11, 45] and to discard the formation of silver particles from ionic silver [22].

In this study, the method used by Jiménez-Lamana et al. [15] was applied. The centrifugation conditions used in [15] were 3000 rpm for 15 min; however, in our study, it was observed that using these conditions, part of silver was retained in the solid. Therefore, centrifugation and sedimentation were compared as separation methods for the alkaline extracts of both animals to study the possible silver losses by ICP-MS. The results are shown in Table 1. With centrifugation, a loss of Ag is observed obtaining an amount of total Ag between 7 and 15%, while with sedimentation, the released silver increased to 30–42%. This agrees with the bibliography [28, 29], in which a loss of Ag in the supernatant is reported. So, sedimentation was chosen as the separation method for the extraction of faeces.

The results obtained for the alkaline extraction with the sedimentation process are shown in Fig. 1. For pigs, only $42 \pm 11\%$ of the total Ag is extracted. For chickens, the Ag extracted is $40 \pm 7\%$. This low percentage of extraction is caused by remaining residues of the indigested faeces which act like a “trap” for the silver forms, preventing their complete extraction [42]. The alkaline extracts were also analysed by SP-ICP-MS. The transport efficiency was measured in ultrapure water and, also, in the presence of 1% TMAH. The two gold calibration curves have shown a difference between the slopes applying the student *t* test; consequently, the transport efficiency used was calculated using 1% TMAH. The calculated transport efficiency values were slightly different giving $7.22 \pm 0.03\%$ for water calibration and $8.63 \pm 0.10\%$ for TMAH calibration. The percentage of the particles released was lower than 1% regarding the

total Ag released (the mass distributions are shown in figure S5 in SI). This percentage represents a very low concentration of particles, which was below the limit of detection of the HDC-ICP-MS ($17.0 \mu\text{g L}^{-1}$). That is the reason why in the chromatogram obtained for HDC-ICP-MS for pigs (see figure S6 in SI), only one peak appears at 8.71 min, which corresponded to Ag (I).

Due to the possibility of the precipitation of Ag(I), Clark et al. [23] used a solution of CaCl_2 for the extraction of Ag(I) and AgNPs in fish tissues to avoid the formation of particles of Ag from Ag(I). In our case, the use of CaCl_2 as a stabilizer to extract silver species from faeces was also evaluated and compared to the use of cysteine and Triton. The results are shown in Table 1. For chickens, there was no difference between both extraction stabilizers; however, for pigs, the extraction with CaCl_2 released less Ag. Thus, the nature of the matrix and the initial quantity of silver in the real sample might affect the extraction efficiency. In the distributions obtained by SP-ICP-MS, chicken faeces had similar distributions for both stabilizers (figure S7b in SI) while distributions for pigs (figure S7a in SI) were different. Extraction with CaCl_2 gave smaller particles than cysteine extraction and a smaller number of particles extracted using the same dilution. The low efficiency of extraction using CaCl_2 in pig faeces did not allow detecting enough particles.

TSPP sequential extraction

To obtain the different fractions of silver species, a sequential extraction developed by Hong et al. [30] for spiked soils was studied. In this scheme of fractionation, TSPP, NH_3 and Na_2S were used to separate the different fractions of the Ag: Ag(I), AgNPs, AgCl-NPs and Ag_2S -NPs. The total % of Ag released is shown in Figs. 1 and 4, and the percentage of each species of Ag is presented considering the Ag extracted by the sequential extraction as being the total Ag extracted (100%) (concentration of Ag species released in table T3 in SI). There were differences in the predominant species depending on the animal. For chickens (Fig. 4b), most of Ag was in Ag(I) form (62%), like the results found with alkaline extraction. The rest of the Ag was present in the form of Ag_2S -NPs (21%), AgNPs (8%) and AgCl-NPs (9%). For pigs (Fig. 4a), most of the Ag extracted was in Ag_2S -NP form (44%). Ag(I) represents 34% of the total Ag

Table 1 Effect of the centrifugation, sedimentation and stabilizer on the alkaline extraction efficiency of silver from pig and chicken faeces by ICP-MS

Samples	TMAH, cysteine and Triton		TMAH and CaCl_2
	Total Ag released (%) with centrifugation	Total Ag released (%) with sedimentation	Total Ag released (%) with sedimentation
Pig faeces	14.9 ± 5.3	42.3 ± 11.3	20.5 ± 6.8
Chicken faeces	9.8 ± 0.6	39.8 ± 6.6	40.4 ± 3.3

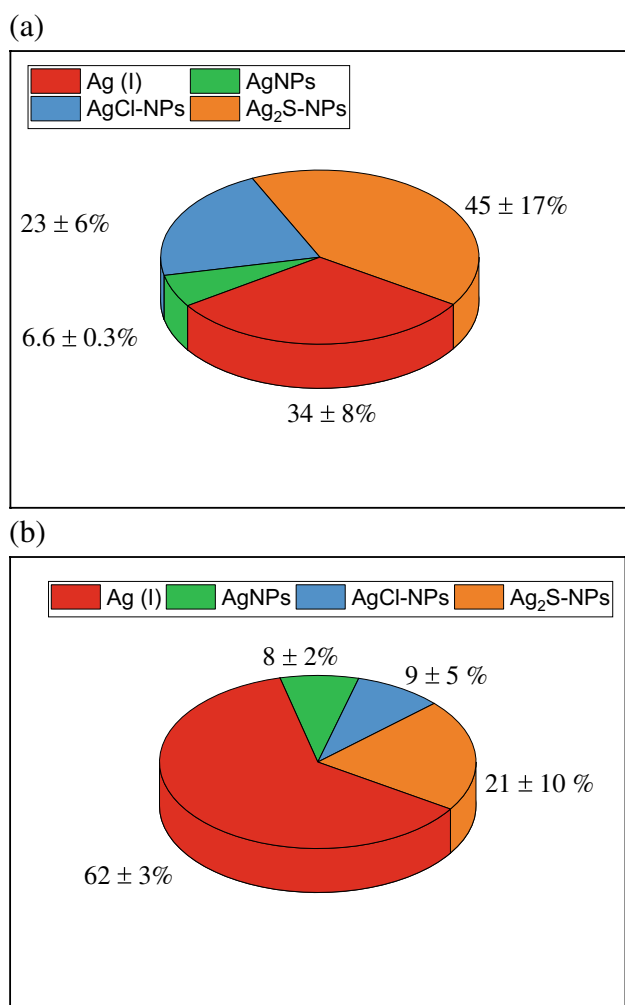


Fig. 4 Percentage of silver forms in faeces of (a) pigs and (b) chickens using the TSPP scheme of fractionation

extracted. The rest of the Ag was in AgNPs form and only 2% of the Ag was extracted as AgCl-NPs. The extraction efficiency obtained by Hong et al. was between 70 and 110% of the total Ag spiked in the soils studied, whereas in this study, the extraction efficiency (Fig. 1) was 23 and 35% for pig and chicken faeces, respectively. These low extraction values can be explained by the fact that the method was developed to extract Ag species in spiked soils, but in our case, the Ag species were already in the faeces; thus, the difference in the matrix (organic matter, pH) might affect the level of extraction. The advantage of the TSPP scheme is the isolation of different fraction and the qualitative characterization of the nature of particles (Ag₂S-NPs, AgNPs, AgCl-NPs). With the sequential extraction, the total Ag released was lower than the amount released by alkaline extraction (see Fig. 1). This is because TMAH is capable to digest the organic matter better than the TSPP and Na₂S, resulting in a higher release of the organic complex of Ag

[28]. Nevertheless, this sequential extraction was useful to know the different forms of Ag.

Behaviour of the different Ag species through the extraction methods

To study the behaviour of Ag species during the various extraction methods employed and to understand the effects of matrix for the different type of samples, the extractions of spiked control samples were carried out using all the mentioned protocols.

Water extraction

Control faeces samples were spiked with Ag(I) and 40 nm AgNPs and then were subjected to the protocol of water extraction. ICP-MS results obtained for total silver determination (figure S8 in SI) are different for pigs and chickens. Meanwhile, for chickens, values were 80–88% for Ag(I) and AgNP spiked faeces; for pigs, the recovery for Ag(I) spiked solution was $1.8 \pm 0.7\%$, and for AgNPs spiked solution was $24.8 \pm 1.9\%$. This difference can be caused by the different compositions of the animal faeces. Moreover, the total Ag released was higher for chickens than for pigs (Fig. 1). The recoveries obtained in the spiked samples agree with that difference. The recoveries obtained by SP-ICP-MS confirm these results. While for chickens, the recovery for Ag(I) was more than 90%, and for AgNPs, more than 77%; for pigs, the recovery was very low (almost 4% for Ag(I) and 23% for AgNPs (see table T4 in SI). There were no particles in the Ag(I) spiked solution for both animals. This indicates that there was no precipitation of Ag(I) during the leaching process. In the AgNP spiked solution for pigs (size distributions in figure S9a in SI), the sizes of the particles found were smaller (28.9 ± 2.5 nm) than the original size (40 nm). This means that the Ag particles oxidize and break during this process, which is reported in the bibliography [31], so it cannot be ensured that the particles which appear in the faeces leaching (Fig. 2a) are the original particles, or they have suffered changes during the process. However, for chickens (figure S9b in SI), the particles found had the same mean size than the original NPs spiked (40.5 ± 0.07 nm), which was also observed in other studies where the equivalent particle sizes of Ag-containing particles extracted from soils with ultrapure water were consistent with the original sizes of the spiked AgNPs sizes [28, 29]. However, although the size was consistent with the AgNPs initially added to the chicken faeces, it is doubtful that the particles remained untransformed during the procedure; thus, secondary precipitates such as the formation of Ag deposited onto organic or clay surfaces, Ag₂S and AgCl may be detected also. The difference in the matrix for both animals is behind this dissimilarity in the behaviour of the spiked AgNPs. These

differences in recovery values were also observed for spiked soils. Whereas Torrent et al. [31] reported recovery values of less than 8% for Ag(I), a small fraction of the AgNPs, Mahdi et al. [7] obtained 39% recovery for the extraction of AgNPs from soils using ultrapure water.

The spiked control faeces after water extraction were also analysed by HDC-ICP-MS. In the chromatograms obtained for the Ag(I) spiked solution (Fig. 5, black line), two peaks appear for pigs (Fig. 5a) and for chickens (Fig. 5b), one of which corresponds to Ag(I), at 9.00 min for pigs and 8.92 min for chickens, and another small peak appears at 7.73 min for pigs and 7.85 min for chickens, which would correspond to Ag bound to kaolin particles, giving sizes greater than 300 nm. This chromatogram was similar to the chromatogram obtained in the original Ag samples; therefore, it was not possible to confirm the presence of AgNPs in the sample,

whereas the presence of Ag bound to kaolin particles or Ag aggregates was confirmed.

Regarding the spike of AgNPs (Fig. 5, red line), two peaks appeared, but they were different for both animals. For pigs (Fig. 5a), one of the peaks corresponded to particles around 60 nm, with a retention time of 8.04 min, and another peak at 9.03 min, corresponding to Ag(I). For chickens (Fig. 5b), one of the peaks was seen at a retention time (8.77 min) close to the retention time of Ag(I) and a previous peak appeared at 7.85 min, the same that was obtained in Ag(I) spiked solution. With these results, the oxidation of the AgNPs in the leaching for both animals would be confirmed, which agrees with what was found by SP-ICP-MS.

Alkaline extraction

For the study of the behaviour of silver during the alkaline extraction, spiked control samples with Ag(I) and 40 nm AgNPs were subjected to this extraction. In the comparison of centrifugation and sedimentation for the alkaline extraction of the spiked faeces, a loss of Ag is observed as in samples. For the spike of Ag(I) in pig faeces, $41.3 \pm 7.2\%$ is recovered with centrifugation versus $77.3 \pm 7.2\%$ recovered with sedimentation. For the spike of AgNPs, the difference is lower, $54.5 \pm 1.2\%$ with centrifugation and $64.8 \pm 2.6\%$ with sedimentation. Same effect for chickens was obtained, although the difference between centrifugation and sedimentation was less than for pigs. The obtained recoveries were $85.0 \pm 0.8\%$ for the Ag(I) spiked sample and $77 \pm 7\%$ for the AgNPs spiked sample. The results confirm that centrifugation affects the recovery of Ag as in samples [28, 29]. The differences in the composition of the faeces of both animals could explain the differences in the recoveries.

The analysis of alkaline extraction by SP-ICP-MS confirmed the presence of particles in the Ag(I) spiked control samples which means that a fraction of the Ag(I) was precipitating during alkaline extraction (figure S10 in SI). Almost 4% of Ag was recovered by SP-ICP-MS in its precipitated form. Vidmar et al. [18] explained that the precipitation of Ag in samples is caused not only because of the presence of TMAH but also because of the components of the samples, specifically, $-\text{OH}$ groups of the proteins present in faeces, which can create an ideal atmosphere for the formation of particles. So, it can be concluded that the precipitation of the Ag(I) present in the faeces contributed to the appearance of Ag particles in the samples.

For the AgNP spiked samples, the size obtained for both animal faeces (40 ± 1 nm) was similar to the original size of the AgNPs (40 nm), and no dissolution was observed. A small shift of the distributions to bigger particle sizes and a decrease in NP frequency was seen (figure S11 in SI) using the particle distribution in ultrapure water as a reference. In the literature, Vidmar et al. [18] observed a slight

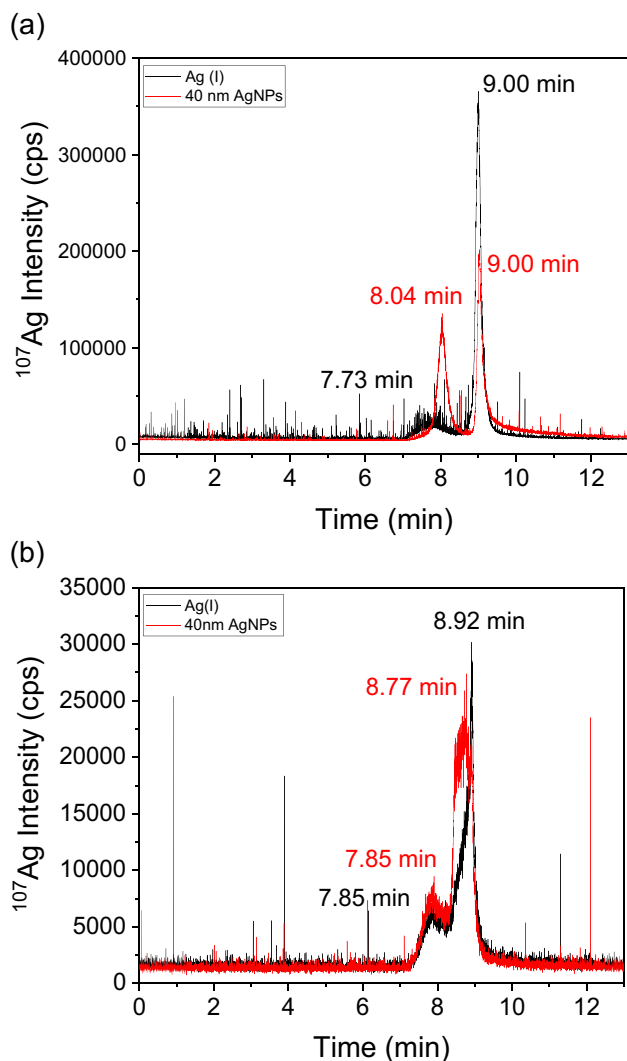


Fig. 5 HDC-ICP-MS chromatograms (a) spike of Ag(I) and 40-nm AgNPs over control pigs faeces (b) spike of Ag(I) and 40-nm AgNPs over control chickens faeces

shift of AgNPs in placental tissue to a larger size using the TMAH extraction; however, the author stated that TMAH alone does not lead to particle formation in the absence of biological matrix; besides, the tissue spiking gave similar size distribution of that of pristine AgNPs. Then, alkaline extraction is a useful extraction method since it does not change the size of the particles which agrees with what is said in the bibliography [11, 22–24], but it is not a suitable extraction method for Ag(I) due to the precipitation observed in the Ag(I) spiked solutions.

TSPP sequential extraction

For the sequential extraction, Ag(I) and 40-nm AgNPs were spiked to control faeces and subjected to the TSPP scheme of fractionation. The recoveries are shown in Table 2. The total Ag recovered was about 80% of the Ag spiked for both suspensions, which is consistent with the article of Hong et al. [30] in which the total Ag and AgNPs spiked in the soil were recovered. In theory, this extraction should mobilise all the Ag in the samples, but faeces subjected to the reported scheme released less than 30% of Ag. Bindings between the different forms of silver and the matrix are stronger in the real sample than when the control sample is spiked, so, for the analysis of an extraction protocol, it is necessary, not only the study of spiked samples because the behaviour could be different from real samples. Other authors found TSPP extraction to be the optimal extraction method for AgNPs; however, it gave lower Ag (I) concentration released, while 25% TMAH gave similar AgNP extraction to TSPP with higher amount of Ag(I) due to more digestion of the solid organic matter [28]. Contrarily, Li et al. [46] also compared the use of ultrapure water, NaNO₃, KNO₃ and TSPP solutions for the extraction of AgNPs from soils and sediments; the optimal method was TSPP extraction. However, we must outline that the samples used in the mentioned studies were spiked soils, while in our study, the samples are faeces collected from intestines of the animals that were orally administrated a silver-kaolin nanomaterial. In our work, low percentages of recovery are obtained.

Table 2 Silver recovery (%) of spiked samples of pig and chicken faeces extracted with TSPP sequential extraction by ICP-MS

Animal	Sample	Ag recovery (%)
Pig	Control faeces spiked with Ag(I)	77.4 ± 1.4
	Control faeces spiked with 40 nm AgNPs	88.3 ± 11.0
Chicken	Control faeces spiked with Ag(I)	95.5 ± 3.4
	Control faeces spiked with 40 nm AgNPs	78.2 ± 5.3

Comparison between the extraction methods

Overall, the comparison of the extraction methods employed throughout this study permitted to conclude that the use of TMAH extraction combined with SP-ICP-MS analysis is useful to characterize the silver-containing particles in faeces. For the AgNPs spiked samples, no transformation or oxidation of the NPs was confirmed. Nevertheless, Ag-containing particles were found in the Ag (I) spiked samples, so we cannot ensure that all the particles which appear in the SP-ICP-MS distribution correspond to Ag particles. Moreover, it can be concluded that the faeces matrix (pig or chickens) in real samples affects the extraction of AgNPs because for spiked control samples, the recoveries were in the range of 65–78%, whereas in real samples were much lower (around 40%).

Although the TSPP method gave lower extraction efficiencies than TMAH method in real samples, it enabled to fractionate the main silver forms in the sample from a qualitative point of view. Different silver species have been found, such as AgCl-NPs and Ag₂S-NPs, in which it is not possible with TMAH method. It is important to underline that the silver species do not behave in the same way when they are in a real sample or when they are spiked on a control sample.

The low percentage of the Ag mobilized made the water extraction a poor quantitative extraction method. Besides, because of the changes suffered by Ag(I) and the AgNPs during the leaching process, it is impossible to ensure the fraction of the silver particles and Ag(I) present in the faeces, as well as to identify the form of Ag. For this reason, water extraction is not a convenient approach for the extraction of silver from faeces, although it is a good option to evaluate the release of total silver from faeces and to determine its subsequent fate and exposure for environmental risk characterization.

The level of uncertainty associated to three extraction methods was evaluated through the repeatability. The repeatability for water extraction was in the range of 6.2–7.6 % ($n = 3$) being similar as for the alkaline extraction with TMAH, cysteine and Triton (between 0.7 and 6.0 % ($n = 3$)). Meanwhile, for the sequential extraction, the repeatability was in the range of 1.8–17% ($n = 3$). The higher values obtained for the sequential extraction are because of all the steps needed in this extraction, which makes the accumulation of errors and causes a decrease into the repeatability.

Conclusions

The comparison of several extraction methods reported in the bibliography was conducted to demonstrate the applicability of these procedures for the extraction of silver species present in the faeces of pigs and chickens fed with a silver-based nanomaterial. Water extraction is

typically used to simulate the release of chemicals from amendments and wastes into the environment. In this study, water extraction resulted in the release of less than 10% of the silver present in pig and chicken faeces, with ionic silver as the predominant form of silver found. The observed changes of silver species during the leaching process make water extraction not a convenient method for the extraction of silver from faeces. Alkaline extraction by using TMAH allows the digestion of organic matter and the extraction of intact silver-containing particles. However, this study has shown that a fraction of the Ag(I) was precipitated during alkaline extraction contributing to the appearance of Ag particles in the samples. TSPP extraction allowed the fractionation of silver into Ag (I) and three particulate fractions, namely, AgNPs, AgCl-NPs and Ag₂S-NPs, which cannot be differentiated by any other extraction method. The relevance of analysing real samples instead of spiked samples has become evident because the silver species extracted did not behave in the same way. In all cases, a small fraction of silver-containing particles was detected. Although each extraction procedure allowed extracting specific silver species, none of these protocols gave a high extraction efficiency to recover all the silver forms in the original samples. These findings reveal the difficulty of characterizing species derived from nanomaterials in real complex samples such as faeces.

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Declarations

Conflict of interest The authors declare no competing interests.

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Nanosilver-based materials as feed additives: Evaluation of their transformations along *in vitro* gastrointestinal digestions in pigs and chickens by using an ICP-MS based analytical platform

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Abstract

The use of a new nanomaterial in the feed chain requires a risk assessment that involves *in vitro* gastrointestinal digestions to predict its degradation and the oral exposure to nanoparticles. In this study, a nanosilver-based material was incorporated to pig and chicken feed as growth-promoting additive and subjected to the corresponding *in vitro* gastrointestinal digestions. An ICP-MS analytical platform was used to obtain information about the silver released in the different digestion phases. It included conventional ICP-MS for total silver determination, but also single particle ICP-MS and coupling to hydrodynamic chromatography for detection of dissolved and particulate silver. The bioaccessible fraction in the intestinal phase accounted for 8-13% of the total silver, mainly in the form of dissolved Ag(I) species, with less than 0.1% as silver-containing particles. Despite the additive behaved differently in pig and chicken digestions, the feed matrix played a relevant role in the fate of the silver.

Keywords: *in vitro* gastrointestinal digestion, Feed additive, Silver; Nanomaterial; ICP-MS

1. Introduction

The introduction of novel nano additives in the animal feed chain requires relevant nano-specific considerations that provide insights into exposure assessment and hazard characterisation of the nanomaterials. In particular, *in vitro*, *in vivo* and toxicological studies should be taken into account to

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Abbreviations: Ag(I): ionic silver, AgNPs: silver nanoparticles, Ag-kaolin: silver-kaolin, ICP-MS: inductively coupled plasma mass spectrometry, SP-ICP-MS: single particle ICP-MS, HDC-ICP-MS: hydrodynamic chromatography coupled to ICP-MS

predict the fate and behaviour of nanoparticles. In this framework, the European Food Safety Authority (EFSA), in its updated guidance on risk assessment of nanomaterials (More et al., 2021), proposes a structured approach for conducting safety evaluations of nanomaterials in food/feed and provides practical recommendations to conduct relevant studies, tests and methods for the identification and characterisation of toxicological hazards. The pathway suggested by EFSA is based on investigating nanomaterials following a scheme of four steps. Once the nanomaterial has been characterized, the second step consists in the evaluation of the degradation/dissolution rate of the nanomaterial under representative conditions of the gastrointestinal tract through *in vitro* digestion models. In this step, if the nanomaterial is rapidly or fully dissolved, it may follow standard safety assessment of conventional/non-nanomaterials rather than further steps of nano-specific testing of the recommended scheme. The third step involves *in vitro* genotoxicity testing which implicates the assessment of cellular uptake, gene mutation, and DNA aberrations. If the nanomaterial is found non-toxic in this step and shows high dissolution/degradation rate during the previous one, then the following step of the approach might be argumented and waived. The fourth step is an *in vivo* study which is recommended for the assessment of the nanomaterial absorption, tissue distribution, accumulation and elimination, as well as dose-finding. If the nanomaterial is found to be slowly eliminated or accumulated in organs, then further tests are required leading to step five of the approach, which includes more targeted and specific *in vivo* studies such as human studies, reproductive and developmental toxicity studies, carcinogenicity, immunotoxicity, neurotoxicity and mutagenicity tests, endocrine impact and gut microbiome effect.

Silver nanoparticles (AgNP), as well as dissolved silver (Ag(I)), have been proven to have antimicrobial properties (Zorraquín-Peña et al., 2020). Application of AgNPs included their incorporation in chicken (Al-Sultan et al., 2022; Kumar & Bhattacharya, 2019; Salem et al., 2021) and pig (Abad-Álvarez et al., 2019; Fondevila, 2010; Fondevila et al., 2009) feeds as an alternative for the use of antibiotics to improve animal growth. Fondevila et al. (Fondevila et al., 2009) reported that low doses of AgNPs added to pig feed reduced coliform bacteria load in the intestine and improved the daily intake and growth of piglets. Due to their antimicrobial properties, silver doped silica nanoparticles were added to chicken diet under a dose level up to 4 mg kg⁻¹ as a safe growth promoter and a promising alternative to antibiotics (Dosoky et al., 2021). AgNPs showed a high antimicrobial activity against the pathogens of poultry farm water and a disinfection potential even though 90% of the nanoparticles were aggregated or sedimented in water (Kumar &

Bhattacharya, 2019). Different authors have demonstrated the antimicrobial activity of AgNPs against pathogens found in poultry that might be a risk to human health. Chickens treated with AgNPs showed decreased mortality and lower colonization when infected with *Clostridium perfringens* (Salem et al., 2021). The addition of 15 nm AgNPs to poultry drinking water reduced the presence of *Escherichia coli* and lowered the chicken mortality rate (Kumar & Bhattacharya, 2019). The presence of silver traces in chicken meat and organs fed with AgNPs was reported to be harmless to human consumption (Aleksandrova et al., 2018). AgNPs capped with acetic acid in drinking water were reported to be a safe antibacterial additive for the increase of broiler productive performance and immune system (El-Abd et al., 2022).

The study of the fate of a nanomaterial after their ingestion to assess its absorption through the intestine is one of the steps of its risk assessment. Different *in vitro* gastrointestinal digestion protocols are described in the literature to study the transformations of AgNPs through the gastrointestinal tract, although all of them are intended for human digestion, while such studies in animals have not yet been conducted to the best of our knowledge. These transformations affect the behaviour and toxicity of silver, as well as the bioaccessibility of the element in the intestine, where it is mainly absorbed. During the digestion process, nanoparticles can suffer different chemical and physical transformations depending on the medium they are in. In a recent review by Qi et al. (Qi et al., 2023), the main transformation of orally ingested AgNPs that might occur during the human *in-vitro* digestion has been discussed in detail. The authors summarized changes that AgNPs could undergo, including agglomeration/aggregation (Pind'áková et al., 2017; Shi et al., 2020) caused by the chloride content of the gastric fluid, oxidative dissolution (Bove et al., 2017; Carnovale et al., 2021) that is related to the acidic pH and the presence of oxygen, sulfuration resulting from the interaction with sulfur-containing molecules after the food fermentation, as well as the corona formation around AgNPs due to the gastrointestinal enzymes and food components. The authors also outlined the confusion faced in many articles about what actually happens in the *in vitro* digestion due to the difficulty of recreating the conditions of the gastrointestinal tract and also because it is not clear whether the silver species transformed/produced during the digestion are absorbed by the body or not. The lack of suitable analytical techniques was considered also a limitation for describing the transformation of ingested AgNPs. Not only the behaviour of AgNPs has been studied, but the transformations of Ag(I) in the presence of human simulated digestion fluids (Abdelkhaliq et al., 2019; Walczak et al., 2013).

Walczak et al. (Walczak et al., 2013) reported the presence of silver containing particles using single particle inductively coupled plasma mass spectrometry (SP-ICP-MS) after the intestinal step, indicating the formation of *de novo* silver particles from Ag(I) as a result of the precipitation of Ag(I) in presence of chloride. Likewise, Abdelkhalik et al. (Abdelkhalik et al., 2019) confirmed the formation of silver particles in the last step of the gastrointestinal simulated digestion, as well as the aggregation and adsorption of 50 nm AgNPs in the digestive matrix. The fate and behaviour of the AgNPs are not only affected by the digestion fluids but also by the food matrix (Kästner et al., 2017; Laloux et al., 2020; Lichtenstein et al., 2015; Walczak et al., 2013) since it can change the ionic strength or the pH of the digestion fluids. Thus, EFSA guidance recommends the study of the impact of food components on the fate of nanomaterials, as changes in the environment of the AgNPs could lead to changes in their behaviour. In the study of Walczak et al. (Walczak et al., 2013), differences in the fate of the AgNPs and Ag(I) were found out during the gastric and intestinal steps due to the presence of proteins. In the absence of proteins, AgNPs oxidized and dissolved in the last step, whereas proteins protected the AgNPs leading to the formation of agglomerates during the second step, which deagglomerated in the third one. The presence of the Ag(I) bound to proteins in the intestinal step was also proved in such study, detecting particles containing silver, chlorine and sulphur by SEM-EDX. A similar behavior was observed by Laloux et al. (Laloux et al., 2020), who applied an *in vitro* human digestion to follow the fate of AgNPs in a standard food matrix. Kastner et al. (Kästner et al., 2017) studied the effect of different food matrices (skimmed milk power, oil and starch), showing that skimmed milk powder stabilised the NPs, whereas oil and starch changed their size distribution, forming aggregates of particles. On the contrary, Lichtenstein et al. (Lichtenstein et al., 2015) revealed that the aggregation of the NPs only happened in the absence of food components, meanwhile in presence of the food, the NPs were protected by the formation of aggregates/agglomerates, most probably because of the non-ionic components, which prevented their formation in acid conditions. Ramos et al. (Ramos et al., 2017) highlighted the different behaviour of 40 nm AgNPs standard in comparison with chicken meat spiked with 50 nm AgNPs. The authors reported that 97% of silver was measured in the intestinal fluid when 40 nm AgNPs were *in vitro* digested, whereas only 57% of silver was recovered from the spiked meat. On the other hand, the detection of silver nanoparticles with smaller sizes was also reported, as well as their dissolution along the digestion steps. However, only SP-ICP-MS was used for detection of particulate silver and the composition of the particles detected was not confirmed, thus reporting only tailed distributions that might correspond to particles containing silver rather than metallic AgNPs.

Whereas harmonized *in vitro* protocol for simulating human digestion have become available as a result of international consensus (Brodkorb et al., 2019; Egger et al., 2016; Minekus et al., 2014), this is not the case for animal digestion. Egger et al. (Egger et al., 2017) developed an *in vitro* digestion method, based on the standardized *in vitro* digestion for humans developed by Minekus et al. (Minekus et al., 2014). The *in vitro* digestion method was evaluated in an inter-laboratory study (Egger et al., 2016) and validated by comparing it with an *in vivo* experiment (Egger et al., 2017), concluding that the *in vitro* digestion method could be used as a robust and simple tool to study the gastrointestinal digestion processes in pigs. Different *in vitro* methods have been used to simulate the digestion of proteins (Bryan et al., 2018) wheat (Lafond et al., 2015) and tritic (Yegani et al., 2013) in chickens, as well as common poultry feed ingredients (maize, soybean meal, sunflower cake and de-oiled rice) (Narasimha et al., 2013). Yegani et al. developed an *in vitro* digestion method to simulate the feed digestion in chickens (Yegani et al., 2013), where incubation times and enzyme concentrations were optimized.

Despite the proposal of using silver based nanomaterials in feed additives, *in vitro* digestion studies for animals are scarce. Abad-Alvaro et al. (Abad-Álvaro et al., 2019) studied the fate of the AgNPs in weaned piglets but the protocol involved only gastric and intestinal steps, observing the formation of silver chloride particles during the gastric step and their subsequent dissolution in the intestinal step.

In this study, a nanosilver material, called silver-kaolin (Ag-kaolin), was risk-assessed under the scope of the EFSA Guidance. The nanomaterial contained AgNPs adsorbed on kaolinite microparticles and it was developed to be used as growth promoter and antibiotics substitute in animal feed. The antibacterial activity and the *in vitro* genotoxicity of the silver-kaolin were conducted in previous studies (Pérez-Etayo et al., 2021; Rodriguez-Garraus et al., 2022). The material was found to have an anti-bactericidal activity against a wide variety of bacterial strains and did not cause DNA damage or gene mutation in mouse lymphoma cells. Following EFSA recommendations, the objective of this work is the assessment of the fate and transformations of silver from the Ag-kaolin additive during the *in vitro* gastrointestinal digestion simulated for chickens and pigs. A platform of analytical techniques, which include ICP-MS, SP-ICP-MS and hydrodynamic chromatography coupled to ICP-MS (HDC-ICP-MS) was used. For validation purposes, the same *in vitro* digestion procedures were applied to standards of Ag(I) and AgNPs spiked to control feed to study the behaviour of these species along the different steps of both *in vitro* digestion procedures.

2. Materials and methods

2.1. Instrumentation

A Perkin-Elmer model NexION 2000 ICP mass spectrometer (Toronto, Canada) was used in standard and single particle detection modes. The sample introduction system consisted of a baffled cyclonic spray chamber and a concentric nebulizer (Meinhard, Colorado, USA). Before analysis, ICP-MS measurement conditions were optimized according to the manufacturer instructions to achieve the maximum sensitivity for ^{107}Ag using a $1\ \mu\text{g L}^{-1}$ Ag(I) standard. SyngistixTM 2.5 (Perkin Elmer, Canada) software and the Nano module, was used for data acquisition and to calculate the size and the number concentration of the particles, as well as the mass concentration of dissolved silver. Table S1 in Supplementary material summarizes the standard and single particle experimental conditions used.

For HDC-ICP-MS, a high-performance chromatographic system Waters 2796 Bioseparations module (Waters Corporation, Milford, USA) was used. HDC separations were performed with a PL-PSDA Type 1 column (Agilent Technologies, Germany) with a nominal separation range of 5-300 nm, a length of 80 cm and an internal diameter of 7.5 mm. The sample flow rate used was $1.6\ \text{mL min}^{-1}$. It was coupled to a Perkin Elmer Sciex model ELAN DRC-e ICP mass spectrometer (Toronto, Canada) as a detector. The outflow of the column was delivered directly to the nebulizer of the spectrometer which was a glass concentric slurry nebulizer with a cyclonic spray chamber (Glass Expansion, Melbourne, Australia). Before the analysis, ICP-MS measurement conditions were optimized to achieve the maximum sensitivity for silver, using a solution with a concentration of $10\ \mu\text{g L}^{-1}$. The experimental conditions of the HDC and the ICP-MS are shown in Table S1.

2.2. Reagents and Materials

Aqueous Ag(I) solutions were prepared from standard stock solution of $994 \pm 3\ \text{mg L}^{-1}$ (Panreac, Barcelona, Spain) by dilution in ultrapure water (Milli-Q Advantage, Molsheim, France) by accurately weighing ($\pm 0.1\ \text{mg}$). Diluted suspensions of silver nanoparticles were prepared from commercial suspensions of silver nanoparticles of 10.3 ± 2.1 , 19.1 ± 3.6 , 39 ± 5 and $59 \pm 6\ \text{nm}$ (Nanocomposix, San Diego, USA). Aqueous gold solutions were prepared from standard stock solution of $999 \pm 4\ \text{mg L}^{-1}$ (Panreac, Barcelona, Spain) by dilution in ultrapure water. Gold nanoparticles of $47.8 \pm 1.8\ \text{nm}$ (Nanocomposix, San Diego, USA) with

a concentration of 52 mg L⁻¹ were used as size standard. Nacid (69/70%, J.T. Baker, Phillipsburg, USA) and hydrochloric acid (69/70%, J.T. Baker, Phillipsburg, USA) were used for acid digestions of feeds.

For the pig *in vitro* digestion, pepsin from porcine gastric mucosa stock of 3200-4500 U mL⁻¹ protein (Sigma Aldrich, St. Louis, USA); pancreatin from porcine pancreas based on trypsin activity (Sigma Aldrich, St. Louis, USA) and bile bovine B3883 (Sigma Aldrich, St. Louis, USA) were used to prepare the correspondent enzymes solutions. Potassium chloride (KCl) (Sigma Aldrich, St. Louis, USA), potassium dihydrogen phosphate (KH₂PO₄) (Sigma Aldrich, St. Louis, USA), sodium bicarbonate (NaHCO₃) (Sigma Aldrich, St. Louis, USA), sodium chloride (NaCl) (VWR Chemicals Prolabo, New Jersey, USA), magnesium chloride (MgCl₂·6H₂O) (Fisher, Leicestershire, UK), ammonium carbonate ((NH₄)₂CO₃) (Fluka BioChemika, Buchs, Switzerland), calcium chloride (CaCl₂·2H₂O) (Fisher, Leicestershire, UK) were used to prepare the simulated fluids. Table S2 shows the concentrations of the different salts to prepare the digestion fluids. Sodium hydroxide (NaOH) (Prolabo, Barcelona, Spain) and hydrochloric acid (37%, J.T. Baker, Phillipsburg, USA) were used to adjust the pH of the fluids.

For the chicken *in vitro* digestion, sodium phosphate monobasic monohydrate (NaH₂PO₄) (Sigma-Aldrich, Germany), sodium phosphate dibasic (Na₂HPO₄) (Sigma-Aldrich, Germany) and Milli-Q water (Milli-Q Advantage, Molsheim, France) were used to prepare the digestion juices. Sodium hydroxide (NaOH) (Prolabo, Barcelona, Spain) and hydrochloric acid (37%, J.T. Baker, Phillipsburg, USA) were used for pH adjustment. The enzymes used were a pepsin solution (25 mg mL⁻¹; P-7000, Sigma-Aldrich) and a porcine pancreatin solution (100 mg mL⁻¹, P-1750, Sigma Aldrich). Table S3 shows the the composition of gastrointestinal digestion fluids of the chicken *in vitro* digestion.

The mobile phase used for the HDC separation contained 0.45 mM sodium dodecyl sulphate (SDS) (Bio-Rad Laboratories, California, USA) and 1 mM D-Penicillamine (PA) (Sigma Aldrich, St. Louis, USA) in 1 L of ultrapure water. A sodium hydroxide solution and a nitric acid (69/70%, J.T. Baker, Phillipsburg, USA) solutions were used to reach pH 7.5.

The nanosilver based material (Ag-kaolin) was provided by Laboratorios Enosan S.L. (Spain), This material was characterised by field emission scanning electron microscopy (FESEM) to obtain the and size distribution of AgNPs. The diameter of the AgNPs seen by TEM in the nanomaterial ranged from 2 to 100

nm with an average diameter of 27 nm (see Fig. S1 in SI). This nanosilver material was added to the feeds used in this study at 0.2 %(m/m). The composition of the pig and chicken feeds is shown in Tables S4 and S5 in SI, respectively.

2.3. Procedures

2.3.1. Determination of total silver in feeds

For the determination of the silver content in feeds, the procedure by Jiménez-Lamana et al. (Jiménez-Lamana et al., 2014) was used. 100 mg of feed was weighed and put into digestion vessels, 7 mL of HNO₃ and 3 mL of HCl were added. The digestion was performed in a microwave oven (Mars 6 CEM, Charlotte, North Carolina, USA)) at 200°C and 800 psi for 30 minutes. After cooling, the content of the vessels was transferred to polyethylene tubes and made up to 50 mL with 3% HCl (v/v). Standard ICP-MS was used for the determination of total silver. The calibration curve was performed from 1 to 15 µg L⁻¹ of Ag(I) and 10 µg L⁻¹ Rh was used as the internal standard.

2.3.2. In vitro digestion of pig and chicken feeds

2.3.2.1. Pig feed

The *in vitro* digestion procedure followed was first described by Minekus et al. (Minekus et al., 2014) and optimized for pigs by Egger et al. (Egger et al., 2016). Table S6 summarizes the composition of the simulated digestion fluids. An amount of 0.5 g of feed was weighed and put into polyethylene tubes. For the oral step, 3.5 mL of simulated salivary fluid (SSF) and 0.025 mL of 0.3 M CaCl₂·2H₂O solution were added and then filled with ultrapure water until 10 mL. The tubes were vortex and sonicated to ensure the dispersion of the mixture. Then, the suspension was incubated with shaking (99 rpm) for 2 min at 37°C. For the gastric step, 10 mL of oral suspension was mixed with 7.5 mL of simulated gastric fluid (SGF), 0.005 mL of 0.3 M CaCl₂·2H₂O solution and 1.6 mL of porcine pepsin solution. 1 M HCl solution was added to get pH 3.0 and ultrapure water until 20 mL. The suspensions were vortex, sonicated and then, incubated with shaking (99 rpm) during 2 h at 37 °C. In the intestinal step, 20 mL of gastric solution was blended with 11 mL of simulated intestinal fluid (SIF), 0.04 mL of CaCl₂·2H₂O solution, 2.5 mL of fresh bile and 5 mL of pancreatin solutions. 1 M NaOH solution was added to get pH 7 and ultrapure water until 40 mL. The

suspension was incubated with shaking (99 rpm) during 2 h at 37 °C. At the end of each step supernatants were obtained by centrifugation (5000 rpm 17 min) containing Ag-kaolin particles below ca. 1 µm, which were stored at 4°C for further analyses. All experiments were performed in triplicate.

2.3.2.2. *Chicken feed*

An *in vitro* two-step digestion procedure based on previous protocols for *in vitro* digestibility of feedstuffs was used (Boisen & Fernández, 1995; Yegani et al., 2013). 0.5 g of feed were accurately weighed and put into polyethylene centrifugation tubes. For the gastric step, 25 mL of phosphate buffer (0.1 M, pH 6) was added to each sample. Tubes were gently mixed and 10 mL of HCl (0.2 M) were added to get pH 2.0. Subsequently, 1 mL of a freshly prepared porcine pepsin solution was added. Tubes were vortexed for 1 min to ensure homogenization of the mixture, then bath sonicated for 1 min to avoid NPs aggregation. Mixtures were placed in an incubator at 41°C and shaken at 100 rpm for 2 h. After the gastric step, 10 mL of phosphate buffer (0.2 M, pH 6.8) were added, as well as 0.6 M NaOH to get pH 6.8. Tubes were gently shaken and 1 mL of a freshly prepared porcine pancreatin solution (100 mg mL⁻¹) including amylase, lipase and protease was added. Tubes were vortexed (1 min) and sonicated (1 min), then incubated at 41°C with shaking at 100 rpm for 4h. At the end of each step supernatants were obtained by centrifugation (5000 rpm 17 min) containing Ag-kaolin particles below ca. 1 µm, which were stored at 4°C for further analyses. All experiments were performed in triplicate.

2.3.3. *Analysis of digestion fluids*

The total content of silver in the supernatants from the different digestion steps was determined directly by ICP-MS, using Rh as internal standard. Calibrations were performed using Ag(I) standards prepared in 1% (v/v) HNO₃, since no differences on sensitivity were observed when calibrating in the digestion fluid matrices.

SP-ICP-MS and HDC-ICP-MS were used to obtain information about dissolved and particulate silver. For SP-ICP-MS analysis, the supernatants were diluted with ultrapure water, according to their silver concentrations (Abad-Álvarez et al., 2016), and measured using a dwell time of 100 µs and an acquisition time of 50 s for the standards and spiked samples, that had to be increased up to 300 s for the feed samples to improve the detectability for particles. Dissolved silver was determined from the baseline intensity of

the time scans and calibration with Ag(I) standards. Silver mass per particle and the corresponding distributions were determined from the intensity of the individual particle events by considering the analyte transport efficiency, the sample flow rate and the Ag(I) calibration (Pace et al., 2011). The transport efficiency was calculated by the size method (Pace et al., 2011) using a Au nanoparticle size standard. Sample flow rate was determined gravimetrically.

Quantification of the silver species by HDC-ICP-MS was carried out using Ag(I) standards stabilized with 1 mM PA (Jiménez et al., 2021). Size (hydrodynamic diameter) calibration was performed using silver nanoparticles standards with nominal sizes between 10 and 60 nm and plotting their retention time vs. nominal diameter.

The behaviour of Ag (I) and AgNPs along the *in vitro* digestions was studied by submitting standards (5 mg L⁻¹ of Ag(I) and 1 mg L⁻¹ of 40 nm or 20 nm AgNPs) to the different digestion steps, followed by the analysis by ICP-MS, SP-ICP-MS and HDC-ICP-MS. The effect of the feed matrices on the behaviour of Ag(I) and AgNPs was studied by spiking chicken and pig control feeds with standards, and proceeding as above.

3. Results and discussion

3.1. Silver-kaolin additive characterization

The Ag-kaolin additive consisted of spheroidal metallic silver nanoparticles adsorbed on kaolin microparticles, as shown in Fig. S1 of SI. Information about the material can be found elsewhere (Rodríguez-Garraus et al., 2022) and Table S7 summarizes additional physico-chemical parameters. The silver nanoparticles showed diameters in the range of 2 to 90 nm, with a mean diameter of 27 nm. The total silver content was 8.32 ± 0.35 mg g⁻¹.

Dispersion of the additive in water showed the rapid sedimentation of particles over ca. 2 µm, remaining in suspension polydispersed particles with a mean hydrodynamic diameter of 1 µm within 30 min, when measuring by dynamic light scattering. For this reason, a centrifugation step to remove kaolin microparticles over ca. 1µm was included in the different procedures used to isolate the colloidal fraction of the additive in the supernatants of the suspensions (5000 rpm 17 min).

In relation with the solubility of the material in water, the behavior of the metallic silver nanoparticles was considered, since kaolin is insoluble. After 1 h, 35% of the silver was released from a 1 g L⁻¹ dispersion of additive, remaining the silver concentration (2.69 ± 0.18 mg L⁻¹) nearly constant for longer times, as it can be seen in Fig. S2. When dispersed in water, the additive might release silver nanoparticles as well as ionic silver due to the dissolution of the nanoparticles because of their oxidation or most probably of the dissolution of a complex silver oxide outer coating present in metallic silver nanoparticles (Molleman & Hiemstra, 2015). The analysis of the supernatants by HDC-ICP-MS did not allow to detect particulate forms of silver and a single peak corresponding to dissolved silver was obtained (Fig. S3 in SI). However, silver containing particles were detected by SP-ICP-MS at silver concentrations of ca. 1 ng L⁻¹. The silver content per particle was in agreement with the size of the nanoparticles originally present in the additive (Fig. S4), although the detection of kaolin particles containing silver nanoparticles could not be discarded. In any case, The quantitative analyses of the supernatant by HDC-ICP-MS and SP-ICP-MS, as well as after ultrafiltration (3 kDa pore size) revealed that silver was released in dissolved Ag(I) forms ($95 \pm 9\%$), as it can be seen Table S8 in SI, with less than $4 \times 10^{-5} \%$ (m/m) in particles.

3.1. Behavior of silver species along the pig and chicken *in vitro* digestions

Although only the intestinal step is considered relevant by the EFSA Guidance in relation with the degradation of nanomaterials (More et al., 2021), the oral and gastric steps needs to precede the simulation of the intestinal conditions (only the gastric for chickens) and their study can also provide useful information on the transformations and fate of silver. The *in vitro* digestion procedures for pigs and chickens described in section 2.3.2 were applied to Ag(I) and AgNPs standards as well as to pig and chicken control feeds spiked with the standards. At the end of each step, the suspensions were centrifuged and the supernatants analyzed. Silver present in the supernatants of the intestinal step was considered as the bioaccessible fraction.

As a first step, the behavior of Ag(I) and AgNPs during the *in vitro* digestions was studied through the determination of the total silver contents in the supernatants from each digestion step by ICP-MS. Table 1 summarises the silver fraction found in the supernatants, quoted as recovery, from the silver species standards directly subjected to *in vitro* digestion, whereas those from Ag(I) and AgNPs spiked in control feeds are shown in Table 2.

To follow the transformation of the silver species at the different steps of the gastrointestinal digestions, the supernatants were also analyzed by HDC-ICP-MS and SP-ICP-MS. SP-ICP-MS was used to check the presence of particulate silver, whereas HDC-ICP-MS allowed to differentiate between dissolved and nanoparticulate silver, since dissolved silver is eluted at the total elution time of the column and nanoparticles are at shorter retention times according to their size.

The recovery of Ag(I) in the gastric step was in the range of 30-65% for both digestion processes, while in the intestinal step phase it increased to 70-80%. The low recoveries of Ag(I) in the gastric step are associated to the presence of chloride and the precipitation of the silver as AgCl (Carnovale et al., 2021), whereas its redissolution during the intestinal step would be favoured by the presence of enzymes, which resulted in the increase of the bioaccessible fraction both for pigs and chickens. In any case, detectable amounts of particulate forms of silver derived from Ag(I) were not found in the supernatants of the digestion fluids when analyzed by SP-ICP-MS.

The *in vitro* digestion of AgNPs standards resulted in different total silver recoveries for each type of *in vitro* digestion. In the case of pigs, recoveries around 70% were obtained both at the gastric and the intestinal steps. However, for the chicken digestion, recoveries below 10% were obtained in the gastric step, which increased up to 30% in the intestinal step. Moreover, in the supernatants from all the digestion steps, the presence of dissolved silver was detected by HDC-ICP-MS, confirming the oxidative dissolution of AgNPs (Fig. 1 for the intestinal step and Fig. S5 in SM for the rest of steps). In fact, although AgNPs were not detected by HDC-ICP-MS in the supernatant from the pig gastric fluid, they were detected in the intestinal step (Fig. 1), in agreement with chloride-mediated agglomeration of the nanoparticles in the gastric step followed by their disagglomeration in the intestinal one, as reported by different author for human *in vitro* digestion ((Bove et al., 2017; Lichtenstein et al., 2015; Walczak et al., 2013)).

In presence of feed matrix, the total silver recoveries for Ag(I) in the different steps of the pig *in vitro* digestion were in the range of 8-13%, much lower than the previous results without matrix. In the case of the chicken *in vitro* digestion, recoveries were also lower although higher than for pigs, with a bioaccessible fraction close to 60%. These results suggest that the feed matrix contributes to retain Ag(I) in the solid fraction decreasing its bioaccessibility. In the case of AgNPs, the presence of the feed matrix contributed to increase the bioaccessible fraction up to 83 and 55% for pig and chicken digestion, respectively. However,

a relevant part of the nanoparticles were oxidized to Ag(I), as observed by HDC-ICP-MS (Fig. 1), despite the presence of the feed matrix affected the retention times of the different peaks. The relevance of the food/feed matrix has also pointed out by different authors in the case of human *in vitro* digestion (Kästner et al., 2017; Laloux et al., 2020; Lichtenstein et al., 2015; Ramos et al., 2017).

The different behavior of silver species along the *in vitro* digestion for pigs and chickens could be explained by the different conditions in each procedure, namely incubation time (6 h for chickens and 4 h for pigs), temperature (41°C for chickens and 37°C for pigs), the use of different pepsins and the absence of bilis in the chicken intestinal fluid.

3.2. Determination of total silver in feeds

The total content of silver in the different feeds under study is shown in Table S9 of SI. Three replicates of each type of feed were analysed by ICP-MS, following the acid digestion procedures described in section 2.3.1. Control feed showed silver contents of 0.1 mg kg⁻¹ or lower, whereas the content of silver in both supplemented feeds was ca. 12 mg kg⁻¹. Procedure recoveries ranged from 88.2 to 92.6 %, whereas recoveries from spiked pig and chicken feed were 83.8 ± 11.3 % and 104 ± 0.30 %, respectively.

3.3. Fate of silver during the *in vitro* digestion of silver-kaolin supplemented feeds

The *in vitro* digestion procedures for pigs and chickens described in section 2.3.2 were applied to feeds supplemented with Ag-kaolin. At the end of each step, the suspensions were centrifuged to remove kaolin microparticles over ca. 1 µm and the supernatants analyzed. Hence, silver could be present in the supernatants in different forms: as metallic silver nanoparticles adsorbed on kaolin microparticles below ca. 1 µm, as free metallic silver nanoparticles, as dissolved forms of Ag(I) from the oxidation of the silver nanoparticles, as other species from the transformation of the previous ones (e.g., silver chloride nanoparticles, aggregates of metallic silver nanoparticles, other Ag-containing nanoparticles).

First, the content of total silver in the supernatants from the different gastrointestinal digestion steps was determined by ICP-MS. Table 3 summarizes the fraction of silver from the supplemented feeds found in the supernatants. The bioaccessible silver fraction at the end of the intestinal step accounted for less 10% both for pig and chicken simulated digestions. It should be noted that the intestinal fluids contribute to

increase the bioaccessible fraction with respect to the silver released at the end of the gastric step, which was below 3%.

As in the case of the previous studies with silver standards, to get a better insight about the silver species released from the Ag-kaolin additive at the different steps of the gastrointestinal digestions, the supernatants were also analyzed by HDC-ICP-MS and SP-ICP-MS to obtain information about dissolved and particulate silver, where particulate silver may also include residual Ag-kaolin microparticles. Total silver by SP-ICP-MS and HDC-ICP-MS during the intestinal step was in the range 8 to 13% for pigs and 12% for chickens confirming the results obtained by ICP-MS.

3.3.1. Occurrence of silver species during in vitro digestion of chicken feed

Fig. 2 (c and e) shows the mass per particle distribution of silver containing particles detected by SP-ICP-MS from the analysis of the supernatants from the gastric and intestinal steps. Their silver concentrations corresponded to 2 and 7 ng L⁻¹, that accounted for 0.02 and 0.06% of the total silver released in respective steps. In addition, the dissolved silver determined by the same technique (0.3 and 1.5 µg L⁻¹) represented the 2.2 and 12.1% of the total silver released in each step, in fair agreement with the results obtained by ICP-MS (Table 3). These results reveal that bioaccessible silver was released from Ag-kaolin mainly as dissolved species from the oxidation of the metallic nanoparticles, although a very small fraction of silver containing particles was also present. Despite the exact nature of the particles cannot be determined by this technique, their mass would be equivalent to silver nanoparticles of 31-34 nm in diameter.

Due to the low amount of silver released from the gastric step, only the intestinal fluid could be analyzed by HDC-ICP-MS. Fig. 3a shows the chromatogram obtained from the supernatant of the intestinal step, which shows a peak at 8.67 min. This retention time is in agreement with the elution of Ag(I) in the presence of the digested feed matrix, as it is shown in Fig. 1b. Another small peak appeared approximately at 8 min which was suspected to correspond to a particulate form of silver, however, its specific nature was unknown. It could correspond to 20 nm AgNPs ($t_R = 7.92 \pm 0.08$ min) resulting from the degradation of the original NPs present in the nanomaterial or to another particulate silver form. In any case, the HDC-ICP-MS chromatogram confirmed the presence of dissolved silver in the bioaccessible fraction, whose quantification corresponded to 12 ± 4 % of the total silver released in the intestinal step, in agreement with

the results obtained by ICP-MS and SP-ICP-MS (Table 3). The oxidation of AgNPs to Ag(I) during the gastrointestinal digestion was confirmed by the analysis of 40 nm AgNPs and control feeds spiked with 40 nm AgNPs (Fig. 1d).

3.3.2. Occurrence of silver species during *in vitro* digestion of pig feed

Fig. 2 (a, b and d) shows the mass per particle distribution of silver containing particles detected by SP-ICP-MS for the analysis of the supernatants from the oral, gastric and intestinal steps. These results revealed that the levels of particulate forms of silver in the different digestions steps were very low and acquisition times had to be increased from 1 to 5 minutes and the particles detected corresponded to silver concentrations of 1 and 2 pg L⁻¹ in the oral and gastric steps and below the limit of quantification in the intestinal one, which accounted for around 0.00001% of the total silver released, with a most frequent silver mass per particle of ca. 60 ag. In addition, the dissolved silver determined by the same technique (0.26, 0.30 and 1.70 µg L⁻¹) represented the 2.1, 2.7 and 13.5% of the total silver released in each step. These results reveal that the bioaccessible silver was released from Ag-kaolin mainly as dissolved species from the oxidation of the metallic nanoparticles, as in the case of chicken, whereas the fraction of silver containing particles was even lower.

The HDC-ICP-MS chromatograms obtained from the supernatants of the different steps of the *in vitro* digestion are shown in Fig. 3b. Peaks from 8.40 up to 9.25 min were obtained. Comparison with the elution of Ag(I) and 20 nm AgNPs standards and supernatants from the *in vitro* digestion of these spiked species alone and in the presence of control feed (Fig. 1a and 1c) confirmed that particulate silver was not detected, in agreement with the low levels observed by SP-ICP-MS. As a consequence, all the peaks recorded corresponded to Ag(I) species, being delayed or splitted with respect to the elution of the standard due to the complexation of the ionic silver with different compounds from the feed or digestion fluids and the different interaction of these species with the stationary phase of the column. Even the two peaks observed from the intestinal step were associated to Ag(I), as it was confirmed by the analysis of spiked control feed (Fig. 1). The quantification of the chromatograms showed that 2.2, 3.3 and 9.0% of the total silver was released in each step, in fair agreement with the results obtained by ICP-MS (Table 3).

4. Conclusions

According to EFSA, the assessment of the dissolution/degradation of nanomaterials in conditions representative of the gastrointestinal digestion is one of the steps for the risk assessment of nanomaterials to be used as feed additives. The fate and transformations of silver nanoparticles from a Ag-kaolin additive, incorporated as a growth promoter in the feed formulations of chickens and pigs, was studied following two *in vitro* digestion models that mimic the gastrointestinal digestion of these animals. Since the additive consisted of metallic silver nanoparticles adsorbed onto kaolin microparticles, the exposure to the nanoparticles should involve their release from the matrix with minor degradation. Due to the different processes that silver, both metallic and ionic, can undergo under gastrointestinal digestion, the silver present in the liquid phase of the intestinal step was considered as the bioaccessible fraction and analyzed by a number of ICP-MS based methods, which include ICP-MS for total silver determination, but also HDC-ICP-MS and SP-ICP-MS to get insight into the different forms, dissolved and particulate, of silver.

The *in vitro* digestion of feeds supplemented with the Ag-kaolin additive (0.2%) resulted in 8-13% of bioaccessible silver, mainly in the form of dissolved Ag(I) species with less than 0.1% as silver containing particles, while the rest of silver was present in the solid fraction, as adsorbed or precipitated Ag(I) species, agglomerated/aggregated AgNPs or Ag-kaolin particles. Since the EFSA guidance considers a cut-off of 12% (mass based) or less of a nanomaterial present as particles after 30 min of intestinal digestion, a nanospecific risk assessment would not be required for the additive under study. The low bioaccessibility of the silver from the additive is reflected in its low retention in animal tissues, as it has been confirmed by *in vivo* experiments (Zaoui et al, 2023). Under such conditions, most of ingested silver is excreted through the faeces (Jiménez et al., 2023), and the use of slurries and manure as ammendements in agriculture should consider the potential mobilization of silver species in the environment.

CRedit authorship contribution statement

Khaoula Ben-Jeddou: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft. **Mariam Bakir:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft. **María S. Jiménez:** Conceptualization, Investigation, Methodology, Writing – review & editing, Supervision. **María T. Gómez:** Writing – review & editing. **Francisco Laborda:** Writing – review & editing, Funding acquisition, Project administration, Resources.

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.

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Appendix A. Supplementary data

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List of figures

Fig. 1. HDC-ICP-MS chromatograms of Ag(I) (a and b) and AgNPs (c and d) standards (red), subjected to *in vitro* digestion (blue) and spiked in control feed and subjected to *in vitro* digestion (black). Analysis of supernatants from the intestinal step of *in vitro* pig (a and c) and chicken (b and d) digestions. AgNPs: 20 nm in pig digestion (c) and 40 nm in chicken digestion (d).

Fig. 2. SP-ICP-MS mass per particle distribution of silver-containing particles released during the *in vitro* digestion of pig and chicken feed supplemented with Ag-kaolin. (a) oral step (b) and (c) gastric steps (d) and (e) intestinal steps.

Fig. 3. HDC-ICP-MS chromatogram obtained from the supernatant of the intestinal step in the *in vitro* digestion of (a) chicken feed (intestinal step) and (b) pig feed (3 steps) supplemented with Ag-kaolin. Retention times of 40 nm AgNPs (in red) and Ag(I) (in green) correspond to their elution in spiked control feeds.

Fig. 1.

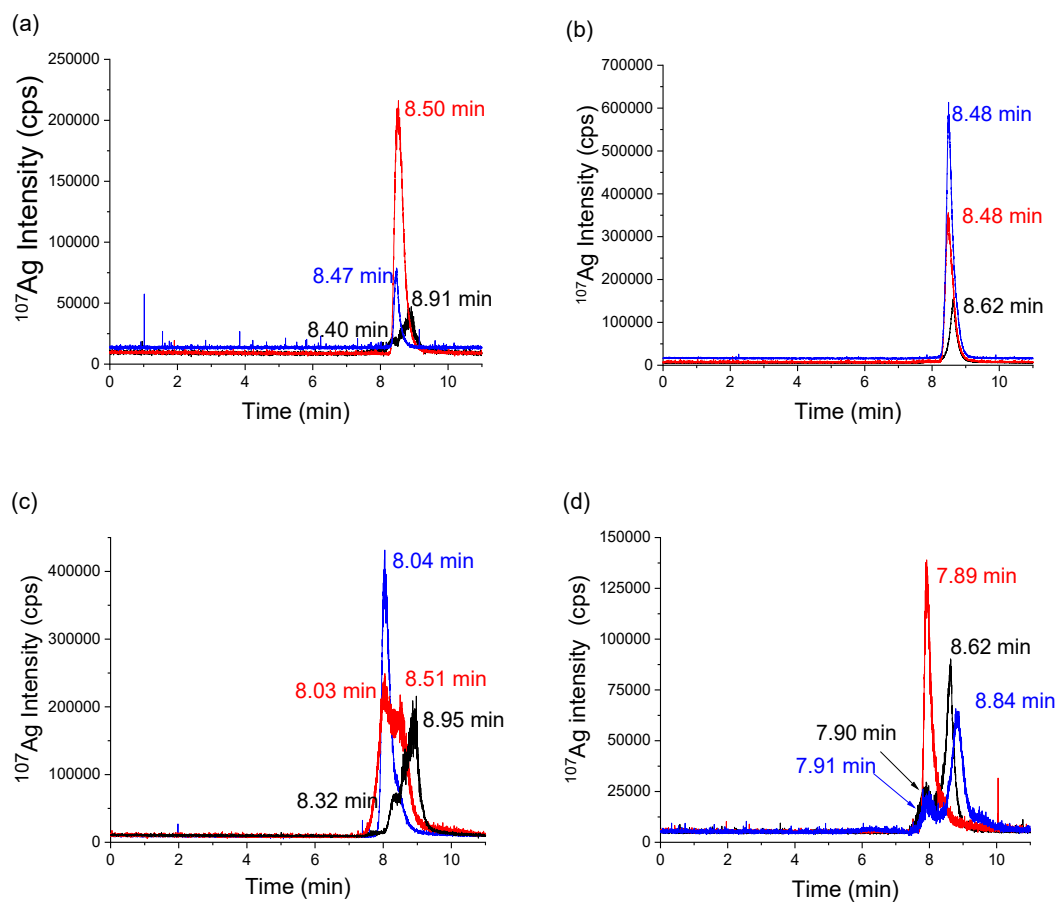


Fig. 2.

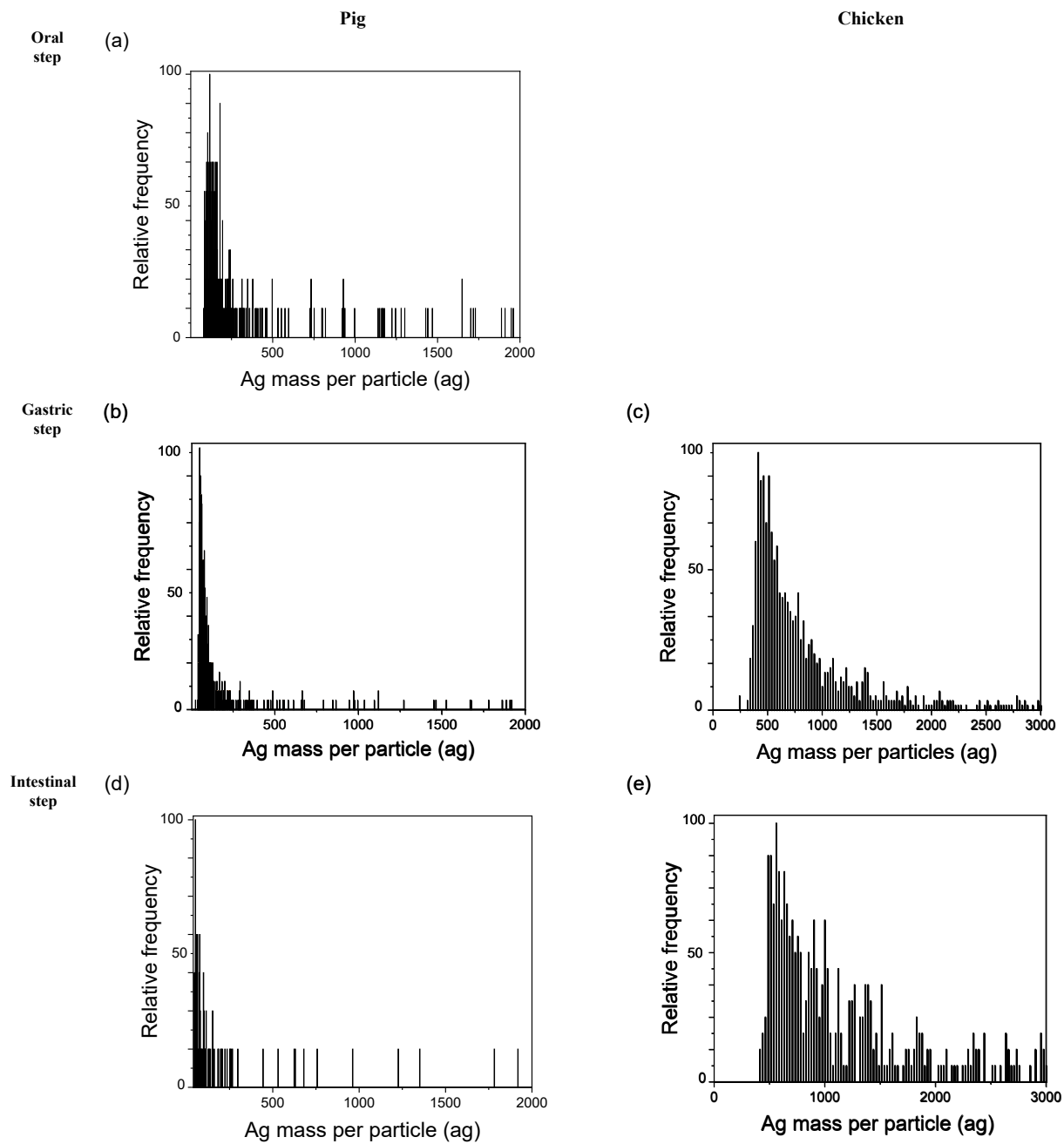
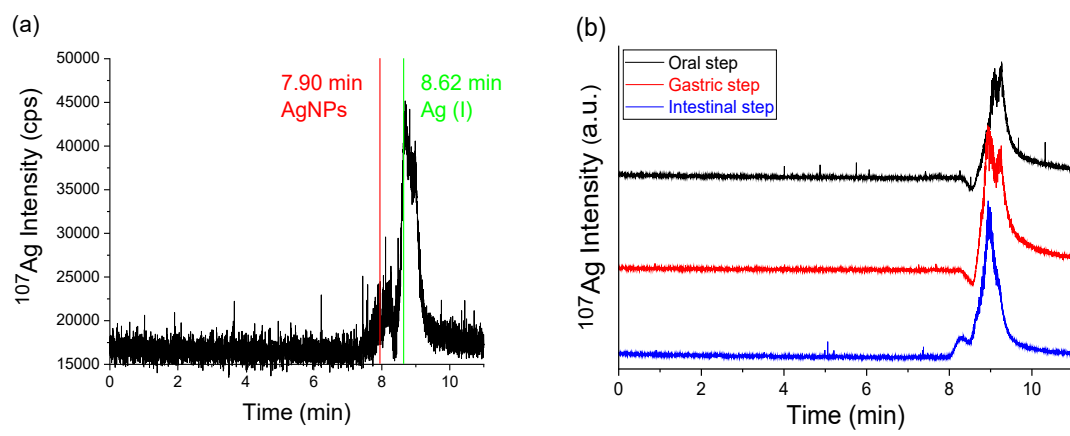


Fig. 3.



List of tables

Table 1. Recovery of Ag (I) and 40 nm AgNPs in each *in vitro* gastrointestinal digestion step for pigs and chickens determined by ICP-MS. Mean $\pm$ standard deviation (n=3).

Animal	Total silver recovery (%)					
	Oral		Gastric		Intestinal	
	Ag(I)	AgNPs	Ag(I)	AgNPs	Ag(I)	AgNPs
Pig	105.3 $\pm$ 7.9	87.1 $\pm$ 5.7	31.2 $\pm$ 1.8	68.0 $\pm$ 18.1	80.8 $\pm$ 1.6	71.5 $\pm$ 3.0
Chicken	-	-	65.4 $\pm$ 1.0	8.7 $\pm$ 0.1	72.9 $\pm$ 9.4	30.5 $\pm$ 0.1

Table 2. Recovery of Ag(I) and 40 nm AgNPs spiked to control feeds in each *in vitro* gastrointestinal digestion step for pigs and chickens determined by ICP-MS. Mean $\pm$ standard deviation (n=3).

Animal	Total silver recovery (%)					
	Oral		Gastric		Intestinal	
	Ag(I)	AgNPs	Ag(I)	AgNPs	Ag(I)	AgNPs
Pig	13.2 $\pm$ 1.2	12.3 $\pm$ 1.1	7.3 $\pm$ 0.2	7.9 $\pm$ 1.0	8.0 $\pm$ 0.1	83.2 $\pm$ 5.1
Chicken	-	-	11.1 $\pm$ 0.1	39.0 $\pm$ 6.4	57.0 $\pm$ 1.0	55.1 $\pm$ 3.1

Table 3. Released silver fraction from feeds supplemented with Ag-kaolin in each *in vitro* gastrointestinal digestion step for pigs and chickens determined by ICP-MS, SP-ICP-MS and HDC-ICP-MS. Mean $\pm$ standard deviation (n=3).

Animal digestion	Total silver released (%)								
	Oral			Gastric			Intestinal		
	ICP-MS	SP-ICP-MS	HDC-ICP-MS	ICP-MS	SP-ICP-MS	HDC-ICP-MS	ICP-MS	SP-ICP-MS	HDC-ICP-MS
Pig	1.84 $\pm$ 0.07	2.13 $\pm$ 0.25	2.20 $\pm$ 0.17	1.78 $\pm$ 0.53	2.67 $\pm$ 0.64	3.26 $\pm$ 0.34	8.33 $\pm$ 0.01	13.5 $\pm$ 1.20	8.95 $\pm$ 0.48
Chicken	-	-	.	2.92 $\pm$ 0.70	2.23 $\pm$ 0.90	-	9.05 $\pm$ 2.00	12.10 $\pm$ 3.10	12.10 $\pm$ 3.90