SILVER NANOPARTICLES-CLAYS NANOCOMPOSITES AS FEED ADDITIVES: CHARACTERIZATION OF SILVER SPECIES RELEASED DURING *IN VITRO* DIGESTIONS. EFFECTS ON SILVER RETENTION IN PIGS

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Abstract

Two different clay nanocomposites, as sepiolite-Ag and kaolinite-Ag, are studied as carriers for silver nanoparticles (AgNPs) oral administration as antimicrobial agent in additives for animal feed. A three-step digestibility assay, corresponding to stomach, small and large intestine simulations, has been followed. Ultrafiltration and asymmetrical flow field-flow fractionation (AF4) coupled to UV-Vis absorption and ICPMS detectors have been used for size characterisation of the silver species released during the *in vitro* digestibility assays. Less than 1% of the total silver is released in the stomach simulation step, probably due to the formation of silver chloride on the nanocomposite surface. In the case of the intestine simulation, silver released increases and tends to form complexes with the enzymes present in the media. A larger amount of silver was released from kaolinite-Ag compared to sepiolite-Ag (17 *vs.* 7 %), probably due to a higher retention rate of silver shown by sepiolite, justified by its sorption capacity and fibrous structure. No evidences were found about a significant release of silver nanoparticles along the *in vitro* assay from any of the two nanocomposites studied. These results have been compared to the silver levels found in liver and faeces from weaned pigs fed with these supplements for 35 days and followed by basal diet free of silver for 4 weeks in an *in vivo* assay. Piglets fed with kaolinite-Ag retained more silver in liver than those receiving sepiolite-Ag, and the opposite was observed in faeces, although differences were not statistically significant. Silver levels found in muscles were below the limit of detection (0.009 μg Ag g^{-1}) in all cases.

Keywords: Ag nanoparticles; digestibility assays; Field Flow Fractionation; ICPMS; clay nanocomposites

1. Introduction

Antibiotics have been used for decades as growth promoters in animal feed to obtain higher productive performances. However, this practise can lead to their retention in animal tissues, with a risk for potential increase of antibiotic resistance processes for consumers of animal products. Following the ban of antibiotics as growth promoters in the European Union [1], the research for alternatives has increased. Organic acids, probiotics, prebiotics, essential oils and plant extracts are some of the most widely used products in poultry and swine production [2][3][4]. In this context, silver has raised as a potential alternative. Silver in its ionic form (nitrates, sulphates or chlorides) is an antimicrobial agent for many pathogens [5][6]. The use of metallic silver in the form of nanoparticles has been also proposed, due to their greater stability, lower dose required, greater antimicrobial activity and lower toxicity for eukaryotic cells [7][8][9]. For instance silver nanoparticles have been added to drinking water or adsorbed in binding agents and included in diets for chickens [10][11] and pigs [12]. The use of silver nanoparticles has shown to be effective in the reduction of potentially pathogenic organisms [6][13], although studies on their effect in animal production are scarce. Since it is expected that silver activity depends on dose and availability though the digestive tract, the form of administration must be considered.

Clays are structural components of soil that have been employed for many years as industrial minerals, with many applications depending on their properties. The most common in the industry are: kaolinites, smectites and sepiolites [14]. Clays have a high metal sorption capacity due, among other characteristics, to their physical and chemical stability, their high surface area and their cation exchange capacity [15][16]. They are used in animal feed for multiple technological (binding, fluidifying and anti-caking), nutritional (increase in the digestibility of nutrients, reduction of transit speed), sanitary (gastric and intestinal protection, prevention against diarrhoea) and environmental applications (reduction of ammonia emissions and bad

odours). The antibacterial activity of clays is based on the strong adsorption of toxins produced by bacteria, which leads to a significant reduction in the adhesion of bacteria to the surface of epithelial cells. Therefore, the composite based on clays and silver nanoparticles may have a synergistic effect [17], which make them a possible alternative as growth promoter additives and antimicrobial in animal feed.

The study of transformations suffered by silver-based nanomaterials along the animal digestion requires the use of diverse approaches. *In vitro* assays assume some simplifications with respect to *in vivo* systems but can reveal some hints about the potential effects or risk in their use. Some studies have used *in vitro* digestion models [18][19][20][21][22][23][24][25][26] to monitor changes of AgNPs after incubation in artificial environments simulating the different digestive sites. AgNPs and $AgNO_3$ have been tested at different steps of the digestion process by dynamic light scattering (DLS) and scanning electron microscopy (SEM) [18][20][22], single particle-ICPMS [18][19], or transmission electronic microscopy (TEM) [23]. AgNPs have been also detected by AF4 coupled to UV-Vis [26] and also quantified by UV-Vis absorption spectroscopy in other studies [22][25][27]. Aggregation of Ag nanoparticles during *in vitro* digestion in presence of food by small-angle X-Ray scattering has been also described [21]. In most of these studies it has been concluded that AgNPs undergo different processes of agglomeration [18][19–21][26] and changes during digestion, so AgNPs would reach the intestine wall. On the other hand, Bove et al. [22] also determined the fraction of free dissolved ions and silver complexes isolated by ultrafiltration (UF) and quantified by ICP-AES, similarly to Wu et al [25] measuring ultrafiltrates by ICPMS. They demonstrated that most of these NPs dissolved as ions, mostly during the stomach phase, and these ions appeared to be bound to the matrix, so exposure levels were expected to be like those observed for silver ions. The lack of standardized procedures for these assays and techniques to measure these processes (dissolution, aggregation, etc.) represent a limiting factor and can explain the discrepancies observed along these studies.

The use of additives for animal feeding based on nanocomposites (in this case particles of kaolinite and sepiolite with silver nanoparticles deposited on the surface) as antimicrobial agents, requires the study of the potential effects on both human health and the environment. In this study, the total silver contents in different organs and tissues of pigs treated with these nanomaterials were determined and related to the different forms of silver found in *in vitro* digestibility assays.

The objective of the present work is to study the transformation suffered by silver nanoparticles deposited on two different clays-based materials used as carriers during the digestion. A three-step enzymatic incubation for the study of the transformations of silver under digestive conditions was proposed. Total amount of silver released and percentage of ionic silver (ultrafiltered through a 3 kDa pore size membrane) were determined by ICPMS. Other silver forms released were characterised by Asymmetric Flow Field-Flow Fractionation (AF4) coupled to ICPMS as detector, together with UV-Vis and Field Emission Scanning Electronic Microscopy (FESEM). A second study, an *in vivo* assay with weaned pigs fed with these two materials was proposed to determine the effect of silver retention in the animal (liver and muscle have been taken as target organs) and elimination by the faeces, so differences between the two nanocomposites in the fractions characterised in the *in vitro* assays could be compared to *in vivo* absorption and excretion values. This is the first time that in vitro and in vivo results are compared to establish the relationship between the processes during digestion (in vitro) with the effects on the animal treated (in vivo assays) with nanomaterials.

2. Material and methods

2.1 Materials

Two different silver nanoparticle-based clays used in animal feed were studied: kaolinite ($Al_2Si_2O_5(OH)_4$) and sepiolite ($Mg_4Si_6O_{15}(OH)_2 \cdot 6H_2O$), both containing silver nanoparticles

(kaolinite-Ag, sepiolite-Ag) and provided by Laboratorios Enosan, Ltd, Spain. These nanocomposites were characterised by FESEM where the laminar structure of the kaolinite microparticles and the fibrous one of the sepiolite were observed (Figure 1). Silver was detected as spheroidal nanoparticles with average diameters of ca. 30 nm for kaolinite-Ag and ca. 25 nm for the sepiolite-Ag. The silver content in clays was determined by F-AAS following an acid digestion, leading to a silver content of 1.07 ± 0.06 % (n=3) (w/w) for kaolinite-Ag and 0.56 ± 0.02 % (n=3) (w/w) for sepiolite-Ag.

2.2 Instrumentation

A Perkin-Elmer Sciex model ELAN DRC-e ICP mass spectrometer (Perkin Elmer, Toronto, Canada) was used throughout. The sample introduction system consisted of a glass concentric Slurry nebulizer and a baffled cyclonic spray chamber (Glass Expansion, Melbourne, Australia). Moreover, a flame AAS (F-AAS) AAnalyst 200 (Perkin Elmer, Toronto, Canada) was used for the determination of total silver content in clays.

The AF4 system used was an AF2000 (Postnova Analytics, Landsberg, Germany). The trapezoidal channel was 27.5 cm in length and from 2 to 0.5 cm in width, and the spacer used for all the measurements was 350 µm thick. An ultrafiltration membrane of polyether sulfone (PES) (cut-off 5 kDa; Postnova Analytics) was used as the accumulation wall.

FESEM images were obtained using a Carl Zeiss MERLIN^{TM} (Nano Technology Systems, Jena, Germany). The microscope was equipped with an energy-dispersive X-ray (EDX) analysis system INCA 350 (Oxford Instruments, Abingdon, United Kingdom). Before FESEM-EDX analysis, samples were coated with a carbon layer to enhance conductivity. Carbon coating was performed with a Leica EM SCD500 (Leica Microsystem, Vienna, Austria) high vacuum sputter coater.

2.3 Reagents

Aqueous silver solutions were prepared from a standard stock solution of 1000 mg L^{-1} (Panreac, Barcelona, Spain) by dilution by accurately weighing (± 0.1 mg). The carrier used for AF4 separation was prepared dissolving the corresponding mass of sodium dodecylsulphate (SDS) (BioRad, California, USA) in ultrapure water (Milli-Q Advantage, Molsheim, France). Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) (Sigma Aldrich, St. Louis, EE.UU.); disodium hydrogen phosphate (Na_2HPO_4) (Sigma Aldrich); hydrochloric acid (Baker Instra Analyzed for Trace Metals Analysis, J.T. Baker, Holland); pepsin (isolated from pig gastric mucosa, Sigma Aldrich); sodium hydroxide (NaOH) (Scharlau, Barcelona, Spain); pancreatin (from porcine pancreas, Sigma Aldrich); ethylenediaminetetraacetic acid (EDTA) (Scharlau) and Viscozyme (Lysing Enzyme from Aspergillus sp., trademark of Novozymes Corp., Sigma Aldrich) were used in the *in vitro* digestibility assays.

2.4. Analytical procedures

2.4.1. Determination of total silver content from clay nanocomposites

100 mg of each material were weighed (± 0.1 mg) and HNO_3 (1:1) (J.T. Baker, Holland) was added, heating and allowing to evaporate near to dryness. The remaining solid was resuspended in conc. HNO_3 and the solution was made up to 15 mL of 10 % (v/v) HNO_3 in a conical tube. The suspensions were centrifuged for 10 min at 3000 rpm. The supernatant was removed, and the solid residue was washed with HNO_3. The supernatant and washing solutions were combined and made up to 50 mL of 10 % (v/v) HNO_3. Samples were diluted 1:10 with 10 % (v/v) HNO_3 prior to the F-AAS analysis.

2.4.2. Determination of silver released from clay nanocomposites

125 mg of kaolinite-Ag or sepiolite-Ag were put into polyethylene tubes filled with 50 mL of ultrapure water or HCl 0.01 M (depending on the release assay) and placed in a rotary tumbler

for 24 hours in the case of ultrapure water or 2 hours in the case of HCl 0.01 M at 29 rpm and room temperature in darkness. Then, 10 mL of the suspensions were taken and centrifuged for 10 min and 20^{o}C at 1430 rpm for kaolinite-Ag and at 1808 rpm for sepiolite-Ag (Heraeus Multifuge X1R, Thermo Fisher Scientific, Walthman, EE.UU.). Centrifugation conditions were set considering the different density of both materials (kaolinite-Ag: 2.6 g cm^{-3}; sepiolite-Ag: 2.0 g cm^{-3}) in order to remove particle sizes larger than 0.5 µm, according to the procedure described by Bolea et al. [28].

2.4.3. In vivo experimental design and sample collection

36 male piglets weaned at 25 $\pm$ 3 days of age and weighing 7.08 $\pm$ 0.03 kg weight were randomly allocated to three groups (n=6) and housed in 100% slotted floor pens (2.0 x 2.0 m) provided with a grow feeder and an automatic drinking device, in a temperature-controlled barn (23-25^{0}C). Procedures were carried out under the Project License PI53/14 approved by the Ethical and Animal Welfare Committee of the University of Zaragoza, Spain. The care and use of animals were performed according to the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes. Piglets received a prestarter (first 14 days after weaning) or starter (from 15 to 35 days after weaning) diets formulated according to their needs. One group of piglets received the feed not supplemented (control), whereas the other two groups were given the feed supplemented with either kaolinite-Ag or sepiolite-Ag (20 mg Ag/Kg feed). After that period, all animals received a common commercial feed from 36 to 62 days after weaning, resulting, on average, 1.19 kg/d on fresh matter basis. The experimental diets were given *ad libitum*, and pigs had free access to fresh water throughout the experiment.

On days 14 and 62 of the experiment, 18 pigs (six per treatment on each day) were slaughtered (average weight of animals at slaughter were 10.1 kg and 35.5 kg respectively) after previous

stunning with CO_2 and after three hours of feed and water deprivation. Representative samples of liver (on both slaughter days), muscle from the *biceps femoralis* (on 62nd day slaughter) and rectal content (on 14th day slaughter) were taken, frozen at -20^{0}C and lyophilized for subsequent Ag analysis to measure tissue retention and faecal excretion.

2.4.4. Determination of silver content of in vivo samples

Lyophilized liver, muscle and faeces samples were ground manually to a particle diameter below 1 mm. A ground sample (200 mg) was weighed accurately (± 0.1 mg) in a microwave digestion vessel. 7 mL of conc. HNO_3 and 3 mL of conc. HCl were added and the digestion was performed at 200 ^{o}C and 800 psi for 30 min. In order to avoid the formation of insoluble AgCl the digestion was performed in the presence of excess of chloride to stabilize silver as $AgCl_3^-$ [29]. After digestion the volume was made up to 50 mL with 3 % HCl (v/v). Total silver content was then quantified by ICPMS.

2.4.5. Digestibility assays: in vitro enzymatic incubation

The *in vitro* three-step enzymatic incubation procedure described by Boisen *et al.* [30] was followed. This procedure was designed for estimating protein/dry matter digestibility in pigs. An amount of 100 mg of kaolinite-Ag or sepiolite-Ag were weighed in 250 mL polypropylene (PP) Erlenmeyer flasks, to get an initial concentration for each clay in the resultant medium of 2.8 g L^{-1} (corresponding to an estimated silver concentration in the digestive system of 28 mg L^{-1}, according to the fixed dosage in feed of weaned piglets (20 mg Ag/kg feed)). In Step 1, 25 mL of phosphate buffer (0.1 M, pH 6.0) and 10 mL of 0.2 M HCl were added to each flask in this order, and pH was adjusted to 2.0; then, 1 mL of a freshly prepared pepsin solution (25 g L^{-1}) was added. Finally, flasks were closed and put into an incubator (OVAN, Barcelona, Spain) at 39^{o}C and stirred at 99 rpm for two hours. The Step 2 consisted of the addition to the mixture from Step 1 of 10 mL of a phosphate buffer (0.2 M, pH 6.8) and 5 mL of a 0.6 M NaOH solution. Then the pH was

adjusted to pH 6.8 and 1 mL of a freshly prepared pancreatin solution (100 g L^{-1}) is added. Flasks were closed and incubated again under continuous stirring at 99 rpm and 39^{o}C for 4 hours. Finally, in Step 3 10 mL of a 0.2 M EDTA solution were added to the mixture and the pH was adjusted to pH 4.8. Finally, 0.5 mL of Viscozyme, a multi-enzyme complex containing cellulases, were added. Flasks were closed and incubated under the same conditions for 18 hours. All the flasks were weighed after the addition of the chemicals in each step to know the incubation volume. The pH adjustments were made with 1 M HCl or 1 M NaOH solutions, depending on the case. *In vitro* assays with an initial addition of 1 mg L^{-1} of silver (I) were also performed to check the recovery of silver during the process. Assays were made in duplicate. At the end of each step, 10 mL of the suspensions were taken and centrifuged to remove particles larger than 0.5 µm. The viscosity and density of the media were previously measured to adjust the speed of the centrifuge. Thus, the *in vitro* assays performed with kaolinite-Ag and silver (I) were subjected to centrifugation for 10 min at 415 x g and 20^{o}C (Heraeus Multifuge X1R, Thermo Fisher Scientific, Walthman, EE.UU.); whereas those with sepiolite-Ag were centrifuged at 666 x g. Samples were diluted with 5 % HCl (v/v) prior to the quantification by ICPMS.

2.4.6. Determination of dissolved silver fraction by ultrafiltration

The dissolved silver fraction was isolated by using Nanosep centrifugal ultrafilter devices (Pall, Ann Arbor, EE.UU.) with cut-off membranes of 3 kDa (estimated to be equivalent to a 2 nm hydrodynamic diameter). Before used, ultrafilter devices were washed twice by centrifugation with 500 µL of ultrapure water. The second wash solution was used to check for any potential contamination. Suspensions from *in vitro* assays previously centrifuged were sonicated for two minutes; and a volume of 500 µL were subjected to centrifugation for 20 min at 9056 x g and 20^{o}C (Heraeus Multifuge X1R, Thermo Fisher Scientific, Walthman, EE.UU.). The ultrafiltered fraction (ca. 500 µL) was diluted up to 5 mL with 5 % HCl (v/v) prior to ICPMS analysis.

2.4.7. Detection of different silver forms by Asymmetrical Flow Field Flow Fractionation (AF4).

100 µL of the *in vitro* suspensions and 50 µL of the silver release suspensions in water (centrifuged fractions < 0.5 µm) were directly injected in the AF4 channel. A 0.01 % (m/v) SDS solution prepared in ultrapure water and adjusted to pH 8 was used as the carrier. Two different crossflow programs, listed in Table 1, were used. The carrier was degassed prior to use by an online vacuum degasser. The eluent was directed from the channel through an UV-Vis diode-array detector (Shimadzu, Duisburg, Germany) recording the absorbance signal in the range from 200 to 650 nm and to the ICPMS spectrometer, used as a silver specific detector.

2.5. *Statistical analysis*

To make a comparison between the variations of silver content in liver and faeces of pigs fed with the two nanocomposites used along this work (sepiolite-Ag and kaolinite-Ag) and the control, a one-way analysis of variance (ANOVA) was applied. Microsoft Excel version Office 365 (Microsoft Corp., KY, USA) was used to conduct statistical analysis. Values were expressed as mean and standard deviation. Remarkable variations were detected at the $p < 0.05$ significance level.

3. Results and discussion

3.1. Characterisation of the clay nanocomposites. Mobilisation of silver from nanocomposites in ultrapure water

The mobilisation of silver from the two clays materials was studied firstly in ultrapure water, following the procedure described in section 2.4.2 and 2.4.6. The percentage of silver in the leachates with respect to the total amount in the composite was similar in both cases (between 40-48%) as shown in Table 2. However, a large difference was observed when comparing the forms of silver in solution. In the case of kaolinite-Ag, most of the silver mobilised (91 ± 5 %) was

found in the ultrafiltrate (fraction with a molecular weight < 3 kDa), which likely corresponds to ionic silver. In comparison, the lower percentage of silver found in the ultrafiltrate of the sepiolite-Ag assay suggests that a significant fraction of silver is mobilised as AgNPs or associated to small particles of sepiolite remaining in solution. This fact could be explained by its higher sorption capacity [31], which may reduce the amount of ionic silver in solution, together with the less effective centrifugation of the sepiolite, caused by its laminar structure, resulting in a large percentage of silver associated to microparticles of sepiolite retained in the ultrafilter.

3.2. Behaviour of silver during in vitro digestion processes. Quantification of silver released.

The potential transformations of AgNPs during digestion is a complex and dynamic process, and the occurrence of agglomeration/aggregation and dissolution should be considered. Hence, a study about the release of silver in an *in vitro* digestibility assay was performed to know the behaviour of the silver-based nanocomposites in each step of the digestion process. It consists of a three-step enzymatic incubation, as a simulation of gastric (Step 1), small intestine (Step 2) and the large intestine (Step 3) digestion processes. As a first approach, the silver mobilised from the two materials kaolinite-Ag and sepiolite-Ag (described in section 2.1) was quantified by ICPMS, as described in section 2.4.5. Table 3 shows the percentage of silver mobilised in each step of the assay with respect to the total amount of silver in the nanocomposites.

Different behaviours between the two additives and between the different steps of the digestive process were observed. Regardless of the composite studied, a low mobilisation of silver in Step 1 was obtained. These percentages of silver released during Step 1 are in good agreement with those obtained when the composites were subjected to a simple simulation of acidic medium in stomach with 0.01 M HCl for 2 hours. (0.9 ± 0.2 % for kaolinite-Ag and 1.2 ± 0.3 % for sepiolite-Ag). Additionally, the formation of AgCl on both clay surfaces was observed by FESEM (Figures

S1 and S2 Electronic supplementary information), which may also justify the low values obtained of silver mobilised during Step 1.

It should be bear in mind that low silver recoveries (51.2±0.5 %, which represents the percentage of silver determined respect to the silver added) were also obtained in Step 1 when silver was added as $AgNO_3$, where the presence of chlorides in the medium can contribute to the formation of AgCl precipitate that might be lost during centrifugation. On the contrary, recoveries close to 100% for $AgNO_3$ were obtained in Steps 2 and 3.

The larger amount of silver mobilised during Steps 2 and 3 observed with both materials could be related to oxidation processes from AgNPs on clays surface or alternatively, the solubilization of AgCl precipitated during Step 1, caused by the addition of enzymes and complexing agents during these steps, being higher with kaolinite-Ag, particularly in Step 2. In any case, the percentages of silver released along these three steps are much lower than the silver mobilised in ultrapure water. This is relevant since comparatively, only a small fraction of the silver is mobilised during the simulated digestion process, which could have a consequence on its bioaccessibility.

3.3 Study of silver species and their evolution during the in vitro digestion processes.

The fraction of silver mobilised could be composed of ionic silver, silver (I) complexes with those species present in the media (enzymes mostly), silver nanoparticles and precipitate silver(I) salts, as well as silver associated to clay microparticles remaining in suspension after centrifugation (sizes smaller than 0.5 µm). Ultrafiltration using a 3 kDa membrane pore size was employed to separate and quantify the fraction constituted by both free silver ions and silver complexes with low molecular weight substances (< 3 kDa). Results, expressed as percentage of silver ultrafiltrated respect to the total silver content, are shown in Table 3. All these values are relatively low (< 4%), being larger in the case of Step 3 respect to Step 2 and 1. Bove et al [22]

also reported low percentages of silver in ultrafiltrates at 3 kDa in the *in vitro* human digestion simulation in the small intestine medium, although they used a reference material NM300k (from the Join Research Centre (JRC), which contains AgNPs of about 15 nm size) for their studies.

To obtain more information about all the species of silver > 3 kDa along the *in vitro* digestion, the suspensions (fraction < 0.5 µm) were analysed directly by AF4-UV-Vis-ICPMS under the conditions described in Section 2.4.7. Flow Field-flow fractionation is a size-based separation technique where separation takes place in a channel without a stationary phase, caused by the action of a crossflow applied perpendicularly to a laminar flow, so small particles elute first in normal mode. The basis of separation and a detailed description of theory can be found in [32]. Program A (see Table 1), optimized for AgNPs size characterisation [33] was used, and the fractograms corresponding to leachates of both nanocomposites are shown in Figure 2. Step 1 (stomach simulation) was not measured given the low percentage of silver solubilized and the low silver recoveries found. Fractograms from kaolinite-Ag showed two non-resolved distributions in Steps 2 and 3, whereas only one peak was observed in the fractograms from sepiolite-Ag. The first distribution in the kaolinite-Ag fractograms could be related to a fraction of silver associated to enzymes present in the media, which would be in good agreement with the absorbance at 280 nm observed, typical of organic matter. Also a recovery assay by addition of $AgNO_3$ to media from step 2 and 3 was made, quantifying the percentage of silver found in the < 3 kDa fraction by ultrafiltration. In step 2, a recovery value as low as 22 ± 3 % was obtained, whereas this value increased up to 67 ± 5 % during step 3. These results suggest that in step 2 silver (I) is likely associated in a large extent to enzymes (which are retained in the ultrafiltrate), whereas in step 3 most of the silver (I) pass through the filter, likely as EDTA complex, not being retained. Moreover, if these fractograms (Fig. 2 a-b) are compared to the ones obtained for a suspension of kaolinite-Ag in ultrapure water for 24 hours (Figure S3 - Electronic supplementary information), just a signal related to the second distribution is detected, confirming the organic

nature of the first silver signal in the digestive media (not present in ultrapure water assay). The lower amount of silver in the sepiolite-Ag media and its sorption capacity [31] could justify the absence of this fraction in the corresponding fractograms.

Suspensions from Steps 2 and 3 of the kaolinite-Ag assays were injected following the crossflow program B (Table 1). This program applied a higher constant crossflow at the beginning to increase the resolution on the separation of the first fraction followed by a linear decay to elute the rest of species injected. Figure 3 shows the corresponding fractograms for kaolinite-Ag under these conditions. As expected, a slight improvement on the resolution of the first fraction was obtained (up to three peaks not resolved are observed in Step 2 fractogram, which corresponds to different forms of silver). In Step 2 fractogram (Fig. 3 a), there is no correspondence between the silver signal and absorbance at 280 nm. Apparently, most of the organic fraction is eluted at the beginning of the fractogram, coeluting with the void peak, whereas the silver is eluted later, showing correspondence with the small shoulder observed at 7 min of the 280 nm absorbance signal. In the case of Step 3, there is coincidence between the maxima of both signals (silver and absorbance at 280 nm), with a peak at 6.4 min, and a peak and a shoulder in the case of UV-Vs signal at 8.0 min. The presence of viscozyme (an enzyme complex containing a wide range of carbohydrases, including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase) in addition to the enzymes from Steps 1 and 2, justify the larger absorbance signal and the silver associated to these species. Therefore, it can be stated that a large fraction of silver released from kaolinite-Ag as Ag(I) would be associated to the enzymatic digestion processes that take place through the digestive tract.

With respect to the second distribution, it could correspond to larger size species according to the elution time, such as silver nanoparticles, although no UV-Vis absorption signal at ca. 400 nm (due to the plasmon resonance of silver nanoparticles) was observed in any case.

Alternatively, it is possible that silver forms insoluble species (such as chlorides) inside the channel. In fact, some studies carried out with AgNPs in an *in vitro* human gastrointestinal digestion model [18] showed the presence of big clusters of 0.2-0.5 µm with chlorine in their composition as revealed by EDX analysis. Kejlova et al. [20] also studied the changes on size and morphology of AgNPs subjected to action of simulated saliva, gastric and intestinal fluids. The authors stated that AgNPs agglomerate and partially react to form AgCl during exposure to gastric fluids, and strong coagulation occurred when AgNPs were mixed with the simulated intestinal fluid supplemented with pancreatin. They concluded that all these changes depended on pH, ionic strength and the presence of proteins in these fluids.

In this case, this silver fraction could be the consequence of an artefact of the technique and formed during the separation process. The collection of this fraction and subsequent analysis by FESEM did not give any further information about its nature, probably due to the low concentration obtained after the separation by AF4. However, this is not something particular of this technique, and other techniques reported in bibliography [34], like DLS, Electronic Microscopy or Single Particle-ICPMS, can also lead to wrong conclusions in such complex media. Besides, differences on the conditions used in the *in vitro* assays described in literature, such as salt content, pH used, presence of different enzymes, or nanoparticles concentration make comparison between results even more difficult.

3.4. Effect of composite nature on silver retained and excreted from in vivo assays

Total silver content in liver and muscle tissues and faeces from animals fed with a diet supplemented with kaolinite-Ag and sepiolite-Ag were determined as described in Section 2.4.4. Average results from the analysis are shown in Table 4. According to these results it can be stated that silver is significantly accumulated in liver in the period when pigs are fed with nanocomposites, compared to control. Also, the kaolinite-Ag provides a higher amount of silver

retained than the sepiolite-Ag, although differences cannot be considered significant (P>0.05 (at 14 days of the beginning of the assay; P values are given in Table 4, comparing values between the materials and treatment days). On the other hand, a decrease in the silver content in the liver of animals subjected to a second diet without silver for four weeks was observed. Differences were significant for kaolinite-Ag (P<0.05 when compare results from day 14 to day 62), but not in the case of sepiolite-Ag (P>0.05). Previous results [12] showed similar levels of silver retention (1.35 and 2.45 μg Ag/g) in the liver tissue of pigs feed with a diet supplemented with 20 or 40 mg of metallic silver as NPs/kg feed for 35 days after weaning respectively.

Determination of total silver content in faeces showed that, although silver was detected in the faeces of control animals, these levels were much lower than those observed for pigs treated with silver and could be attributed to some level of cross-contamination. Moreover, the levels of silver excreted were especially high compared to those retained in the liver tissues. No significant differences were observed in the silver content of faeces depending on the additive used (P>0.05), although these values were higher in the case of sepiolite-Ag considering the average value.

Attending to all these results, a tendency between the *in vitro* results and the silver retained in liver and excreted can be established. Thus, the lower amount of silver detected in the intestinal simulation steps for sepiolite-Ag nanocomposite respect to the kaolinite-Ag levels is also reflected on a lower amount of silver accumulated in liver, as shown by the results from *in vivo* assays on average. In the same way, given the larger proportion of silver released from kaolinite-Ag during the *in vitro* assays, the amount of silver excreted is consequently lower regarding the levels quantified for sepiolite-Ag. However, they should be considered as a first approach since no statistical differences were found when comparing both materials in the *in vivo* assays, so further studies will be needed to confirm these findings. Finally, silver levels in muscle were determined at the end of the experiment (after 62 days) to evaluate the potential ingestion of

silver through the consumption of meat from pigs treated with these nanocomposites. Neither in control nor in kaolinite-Ag or sepiolite-Ag significant concentrations were detected (values under the limit of detection (0.009 μg Ag g^{-1}) were obtained) so silver in muscles were in the same order that those found in control experiments.

4. Conclusions

According to the assays carried out, it can be concluded that the materials used as supports for silver nanoparticles, which are used as additives in animal feed (kaolinite and sepiolite) show differences with respect to the silver mobilised and its forms. There is no evidence about a significant release of silver nanoparticles from any of these two materials along the *in vitro* digestibility assay, in any of the three steps. The formation of silver chloride seemed to be the dominant process during the Step 1 (stomach simulation), which limited the release of silver to the medium. The analysis of the media from intestine simulation Steps (2 and 3) by AF4-UV-Vis-ICPMS allowed to confirm that silver released forms complexes in a large extent with those species present in the medium (enzymes mostly) in the case of kaolinite-Ag. This fraction was not present when sepiolite-Ag was used, likely due to its fibrous structure and sorption capacity. The presence of another form of silver was detected in both intestine simulation steps and with the two materials tested. This fraction would correspond to insoluble silver forms, although their presence in the original medium was not proved, which shows some of the limitations of the techniques used when applied to such complex systems.

Along this study, a tendency between the *in vitro* results and the silver retained in liver and excreted in the *in vivo* assays with weaned pigs supplemented with the silver-based nanocomposites was found. The absence of silver at significant values in muscle tissues makes this technology a potential alternative as growth promoter, although the environmental impact

of faeces should be evaluated, given the silver content found that would limit its land application as manure management practice.

Acknowledgments

This work was supported by the Spanish Ministry of Economy and Competitiveness and the European Regional Development Fund [project CTQ2015-68094-C2-1-R (MINECO/FEDER)] and Gobierno de Aragón (project E29_17R) and FEDER 2014-2020 "Construyendo Europa desde Aragón". The authors would like to acknowledge the use of Servicio General de Apoyo a la Investigación-SAI, Universidad de Zaragoza, for ICPMS measurements and Laboratorios Enosan S.L. for providing the nanomaterials used along this work.

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List of captions

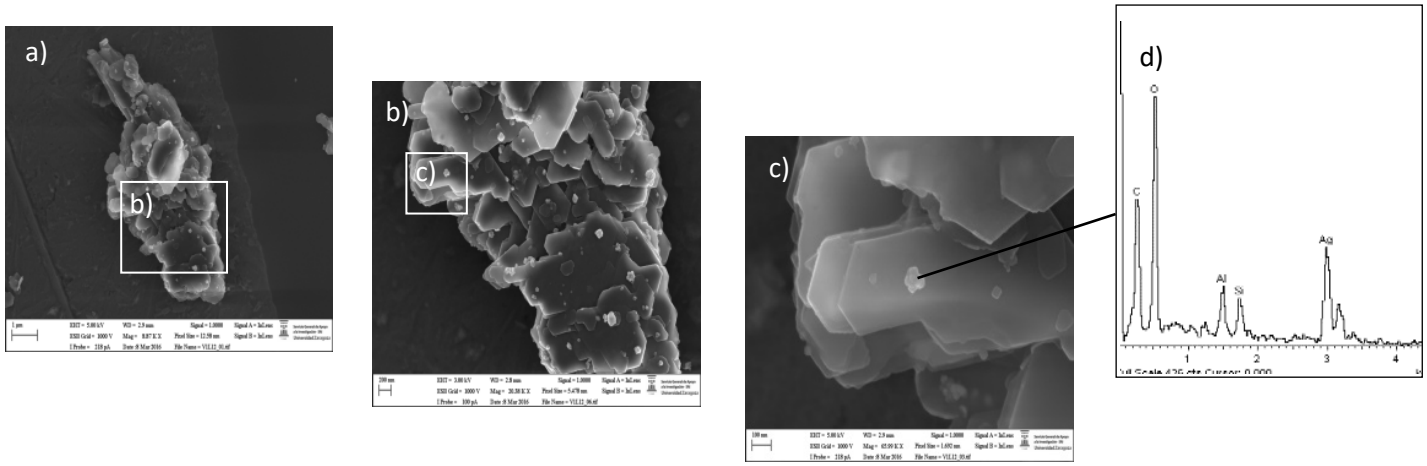
Figure 1. Kaolinite-Ag: a) Microparticle of kaolinite-Ag with silver nanoparticles on the surface (x8870). b) Magnified micrograph from inset in a) (x20380). c) Magnified micrograph from inset in (b) showing silver nanoparticles on the surface of kaolin sheets (x66000). d) EDX spectra from areas containing silver nanoparticles. Silver was found as spheroidal nanoparticles. Sepiolite-Ag: a) Microparticle of sepiolite-Ag with AgNPs on the surface (x2210). b) Surface of a sepiolite microparticle (x47390). (c) Magnified micrograph from inset in b) (x103360) with backscattered electrons. d) EDX spectra from areas containing silver nanoparticles. Silver was found as white spots.

Figure 2 AF4-UV-Vis-ICPMS fractograms of the suspensions from Step 2 (a, c) and Step 3 (b, d) from *in vitro* digestion processes with kaolinite-Ag (a, b) and sepiolite-Ag (c, d). ^{107}Ag ICPMS signal (black line), absorbance at 280 nm (blue line) and at 405 nm (red line). Crossflow program A (green line).

Figure 3 AF4-UV-Vis-ICPMS fractograms of the suspensions from Step 2 (a) and Step 3 (b) from *in vitro* digestion process with kaolinite-Ag. Magnification of fractograms in right upper corner. ^{107}Ag ICPMS signal (black line), absorbance at 280 nm (blue line) and at 405 nm (red line). Crossflow program B (green line).

Figure 1

Kaolinite-Ag



Sepiolite-Ag

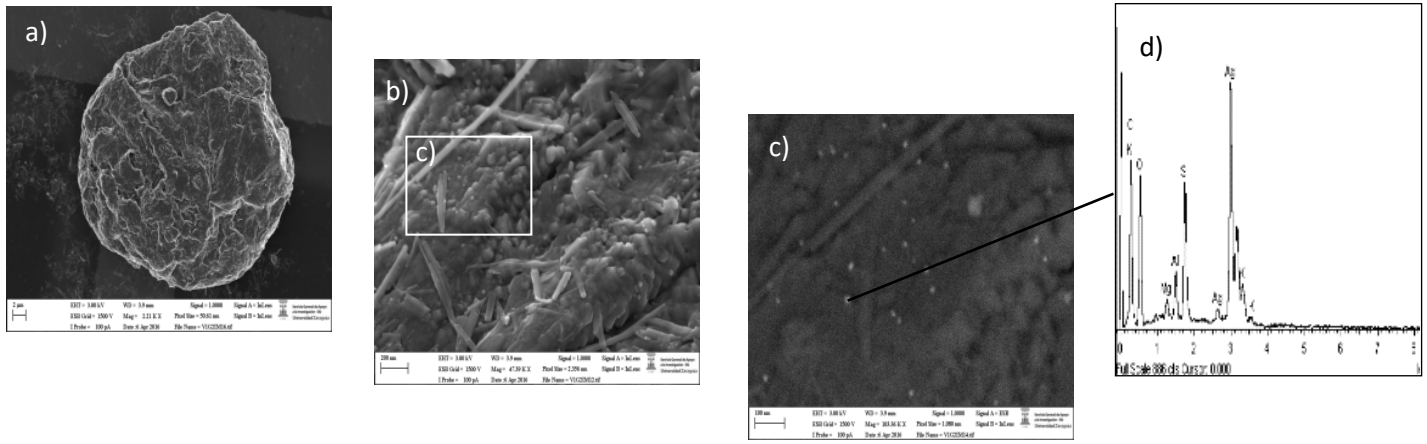
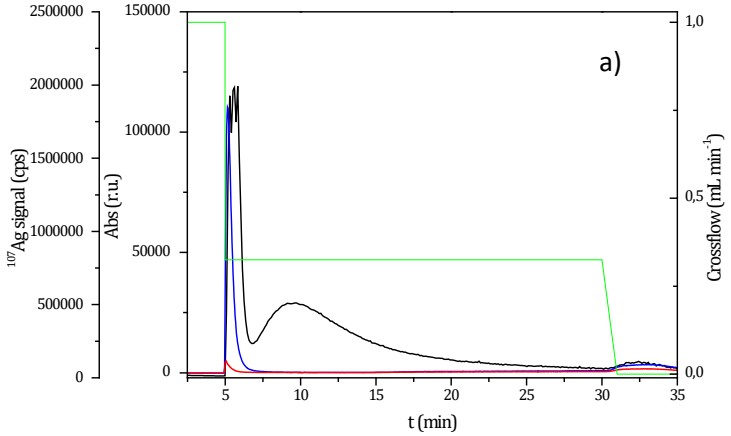


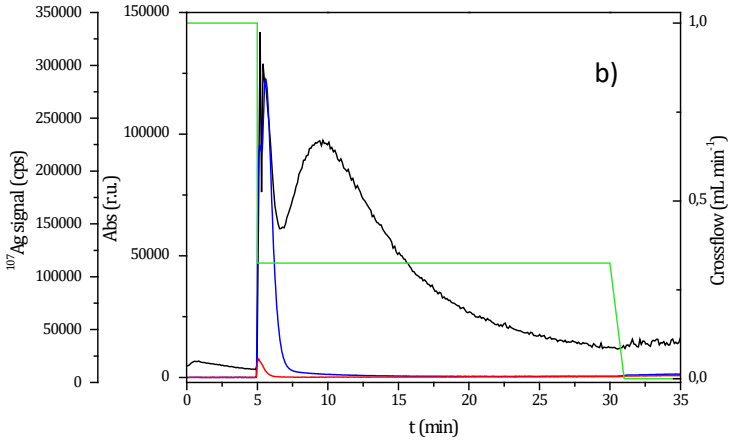
Figure 2

Step 2

Step 3

Kaolinite-Ag





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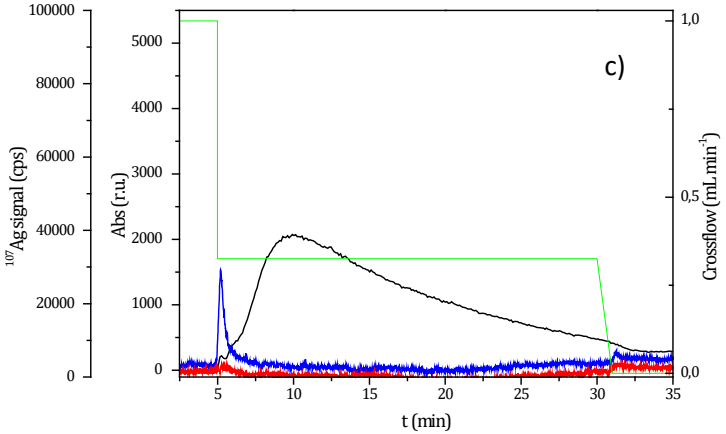


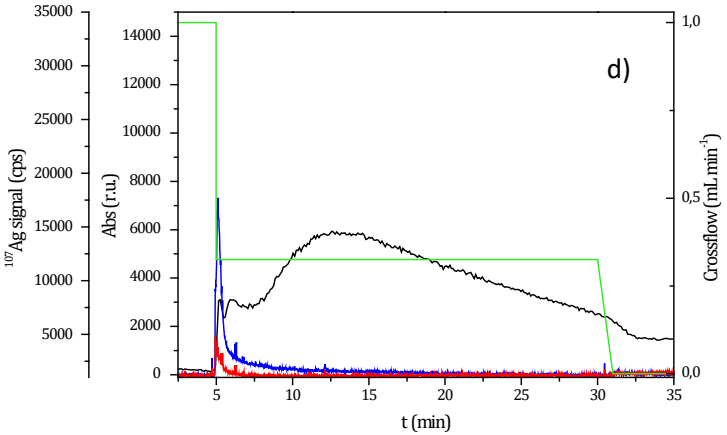
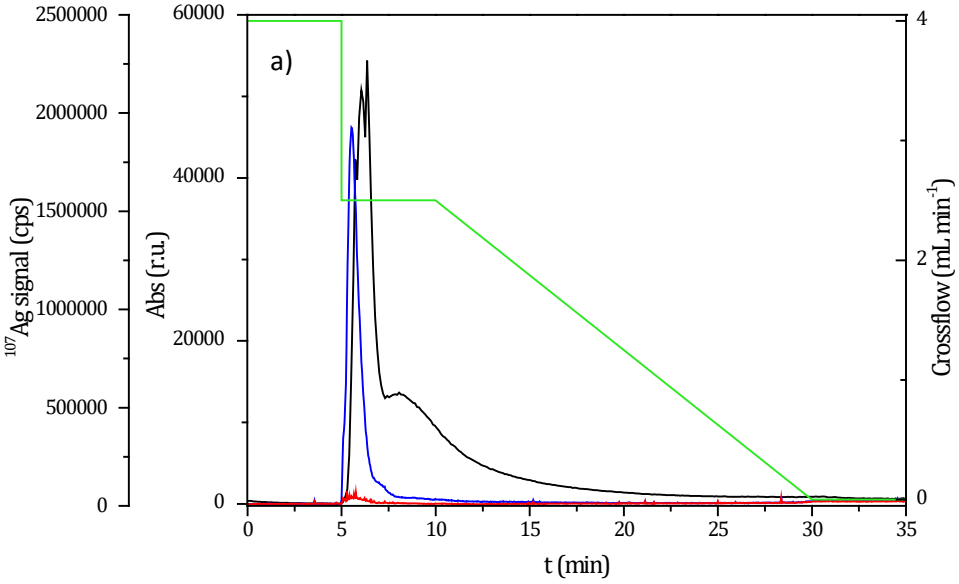


Figure 3


a)
^{107}Ag signal (cps)
Abs (r.u.)
Crossflow (mL min^{-1})
t (min)

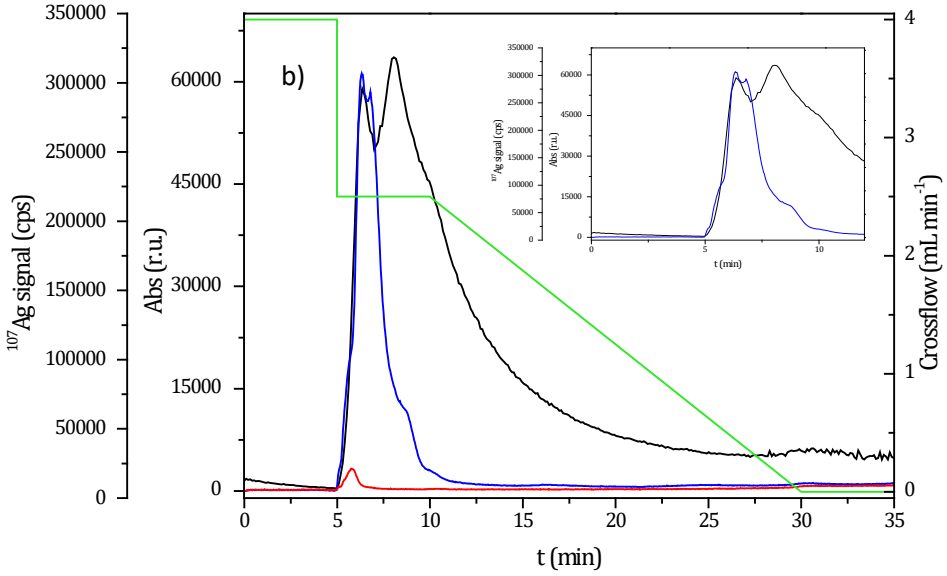

b)
^{107}Ag signal (cps)
Abs (r.u.)
Crossflow (mL min^{-1})
t (min)

Table 1

AF4 crossflow programs. Out flow: 1.00 mL min^{-1}.

Program step	Time /min	Crossflow	
		Mode	mL min^{-1}
Program A: low crossflow			
Injection/focusing	5	(Injection flow, 0.2 mL min^{-1})	1
Separation	25	Constant	0.325
	1	Linear decay	0.325 to 0
	4	Constant	0
Program B: high crossflow			
Injection/focusing	5	(Injection flow, 0.2 mL min^{-1})	4
Separation	5	Constant	2.5
	20	Linear decay	2.5 to 0
	5	Constant	0

Table 2

Percentage of silver mobilised (<0.5 µm) recovered after centrifugation or centrifugation followed by ultrafiltration in ultrapure water respect to the total silver content in the materials. Average ± standard deviation (n=3).

Composite	% Total Ag mobilised	% Ag < 3 kDa of Ag mobilised
Kaolinite-Ag	40 ± 2	91 ± 5
Sepiolite-Ag	48 ± 1	53 ± 2

Table 3

Percentage of silver mobilised (<0.5 µm) in each step of the *in vitro* digestibility assay respect to the total silver content in the materials. Average ± standard deviation (n=3).

Composite	Step	% Total Ag mobilised	% Ultrafiltered Ag mobilised
Kaolinite-Ag	**1**	0.88 ± 0.05	0.38 ± 0.01
	2	17.21 ± 0.13	0.82 ± 0.10
	3	10.16 ± 0.71	1.94 ± 0.05
Sepiolite -Ag	**1**	1.31 ± 0.10	0.78 ± 0.02
	2	4.51 ± 0.25	1.67 ± 0.03
	3	7.31 ± 0.07	3.81 ± 0.16

Table 4

Total silver content (µg g^{-1}) of the lyophilized liver and muscle tissues and faeces from pigs. Average ± standard deviation. P value represents the level of significance. (P<0.05 significant).

	Treatment	Control	Kaolinite-Ag	Sepiolite-Ag	P
		µg Ag g^{-1}	µg Ag g^{-1}	µg Ag g^{-1}	
Liver	**Day 14**	< 0.009 (n = 2)	4.8 ± 1.4 (n = 6)	3.6 ± 1.6 (n = 6)	0.19
	Day 62	< 0.009 (n = 4)	2.8 ± 1.2 (n = 5)	2.2 ± 0.6 (n = 5)	0.35
	P	-	0.04	0.11	
Faeces	**Day 14**	0.05 ± 0.01 (n = 3)	114 ± 29 (n = 4)	141 ± 32 (n = 4)	0.25
	Day 62	n.d.	n.d.	n.d.	-
Muscle	**Day 62**	< 0.009 (n = 6)	< 0.009 (n = 6)	< 0.009 (n = 6)	-

n.d. not determined.

SUPPLEMENTARY INFORMATION FOR

SILVER NANOPARTICLE-CLAYS NANOCOMPOSITES AS FEED ADDITIVES: CHARACTERIZATION OF SILVER SPECIES RELEASED DURING *IN VITRO* DIGESTIONS. EFFECTS ON SILVER RETENTION IN PIGS

I. Abad-Álvaro[1], C. Trujillo[1], E. Bolea[1], F. Laborda[1], M. Fondevila[2], M.A. Latorre[2] and J.R. Castillo[1]

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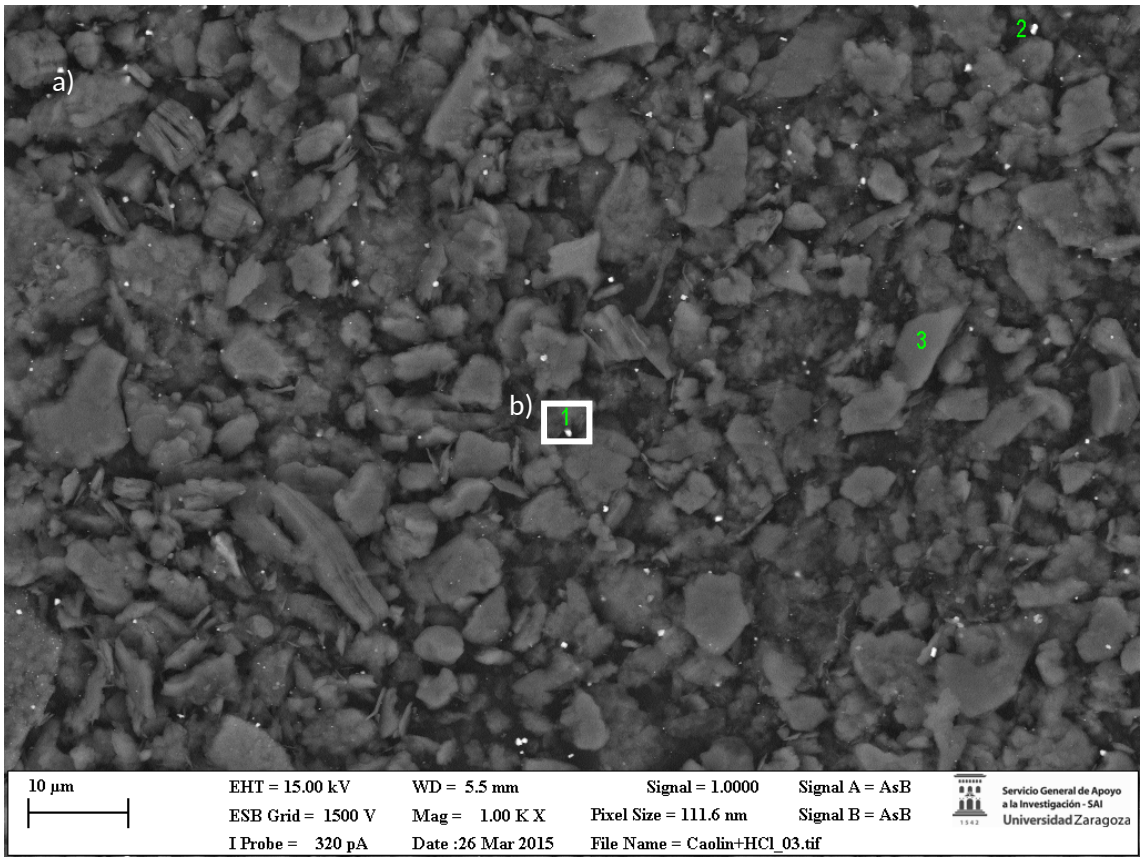
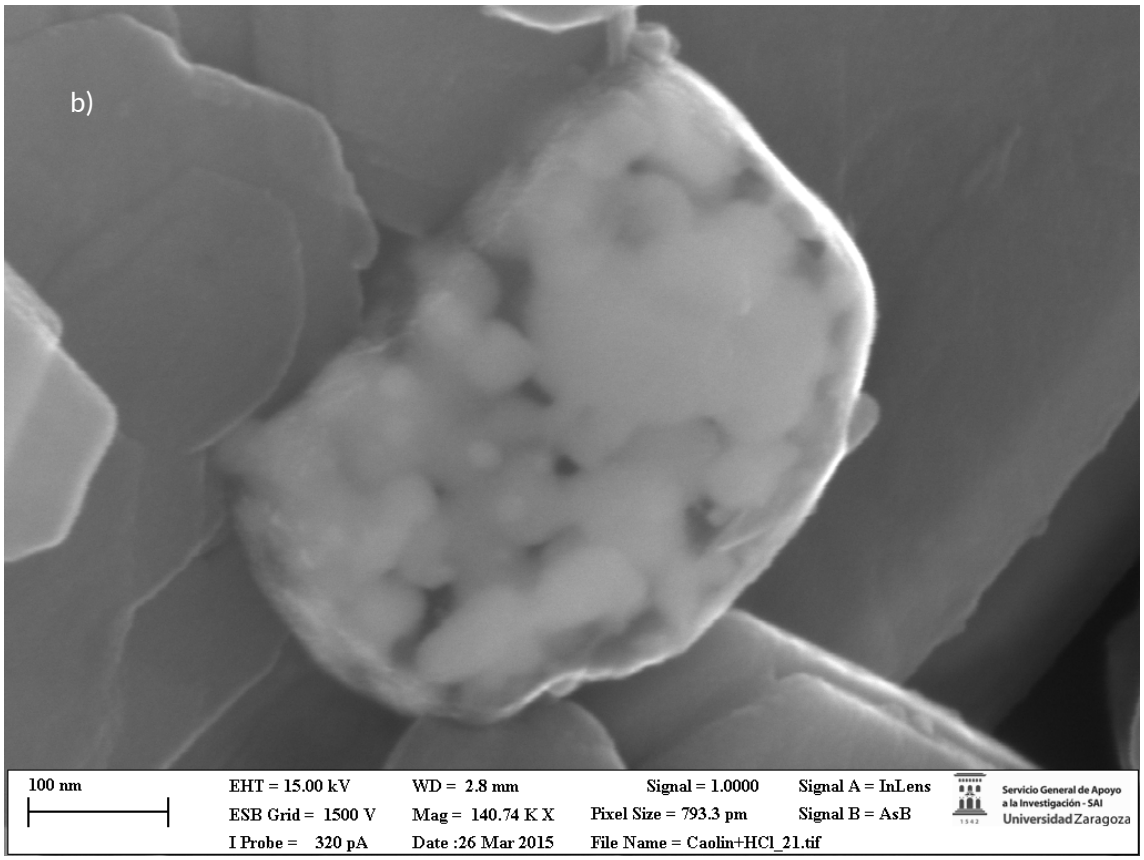




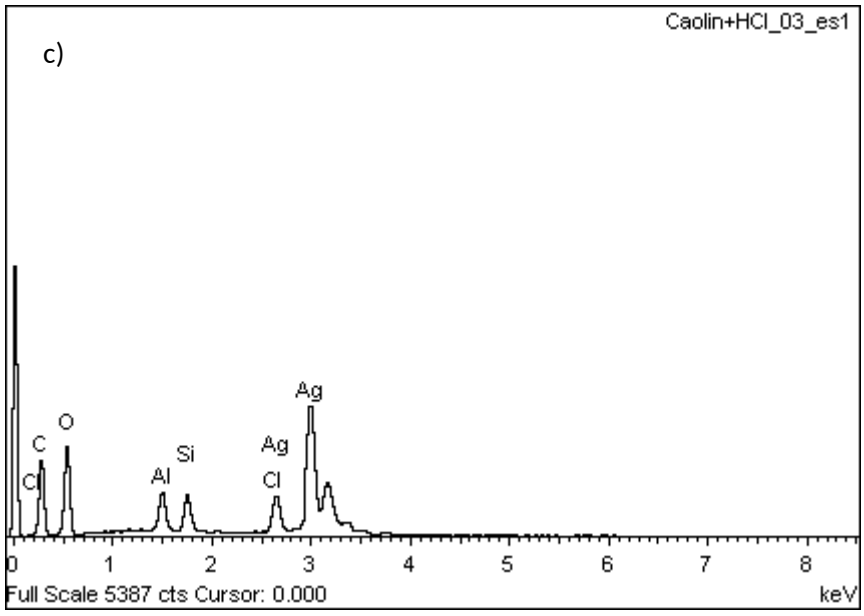
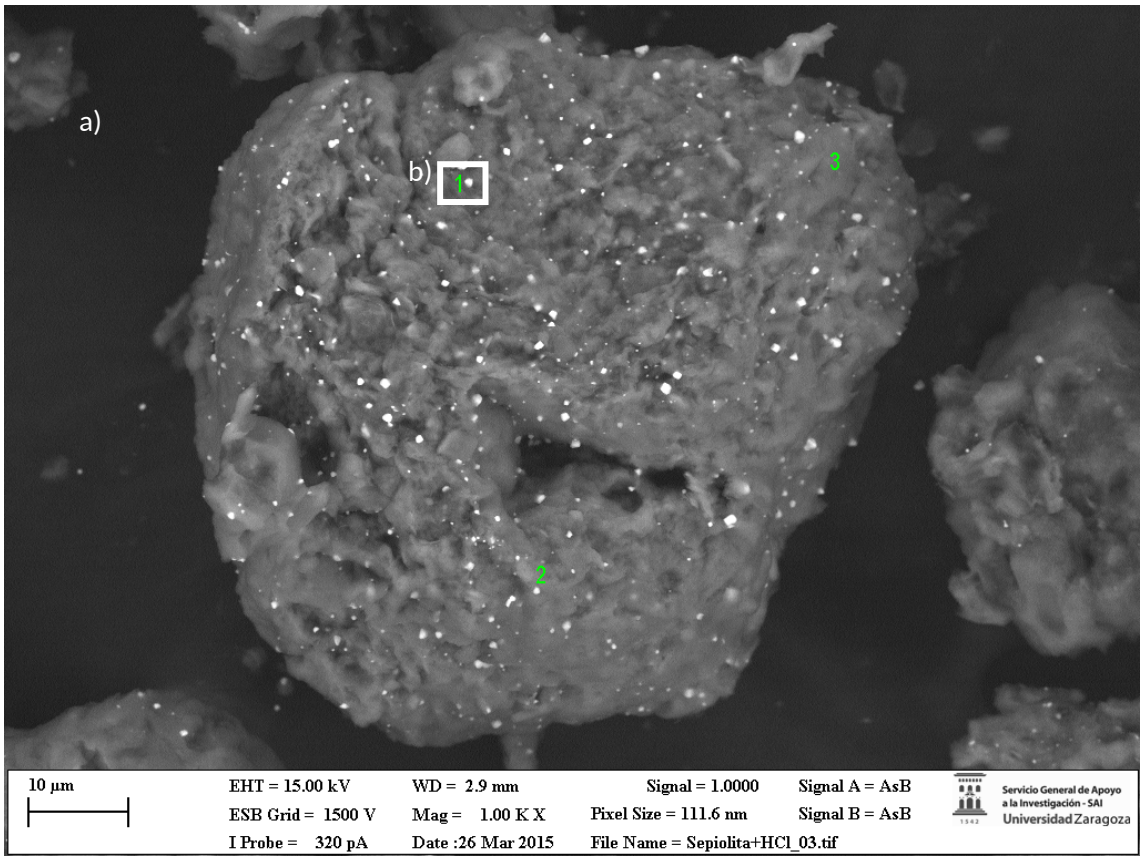


Figure S1 FESEM images. Microparticles of kaolinite-Ag a) with white spots on the surface (x1000) after treatment with HCl 0.01 M. b) Magnified micrograph from inset in a) (x140700). c) EDX spectra from white spots in a), point 1 in green.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b)
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2
3
10 µm
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WD = 2.9 mm
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ESB Grid = 1500 V
Mag = 1.00 K X
Pixel Size = 111.6 nm
Signal B = AsB
I Probe = 320 pA
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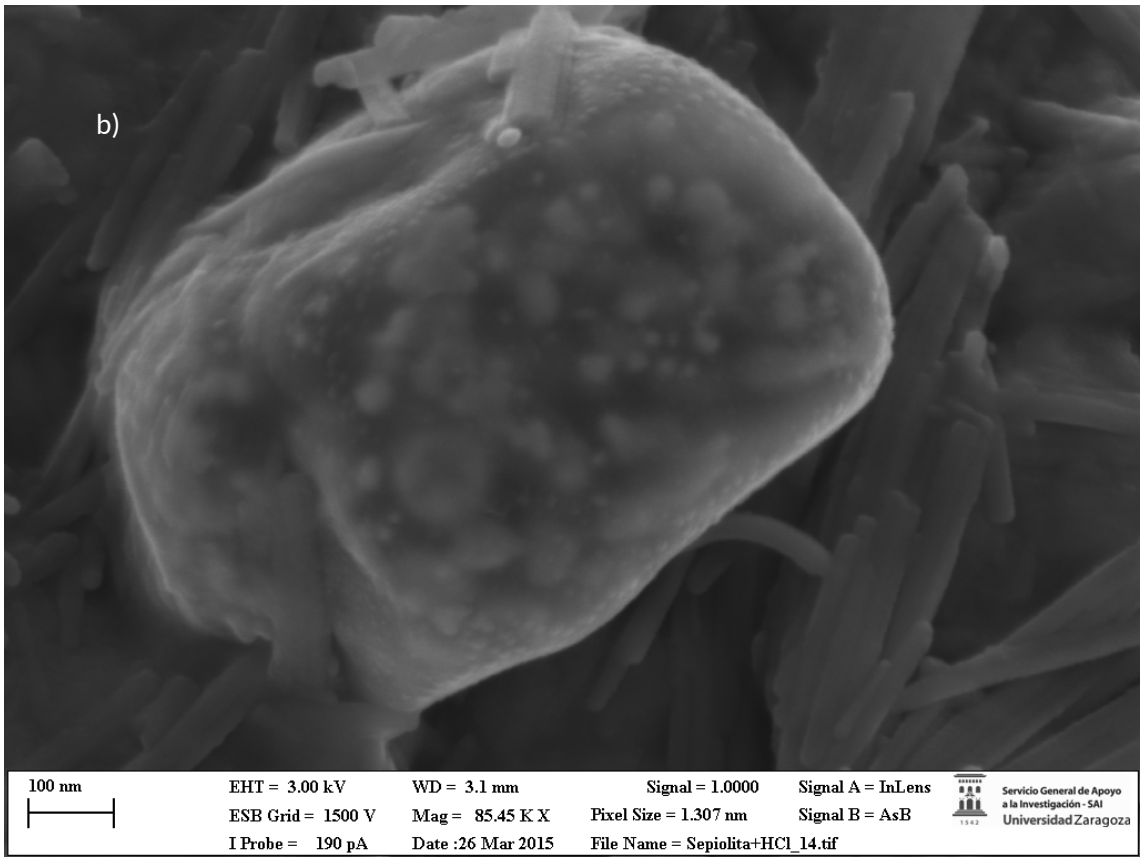
Servicio General de Apoyo a la Investigación - SAI
Universidad Zaragoza

1
2


b)
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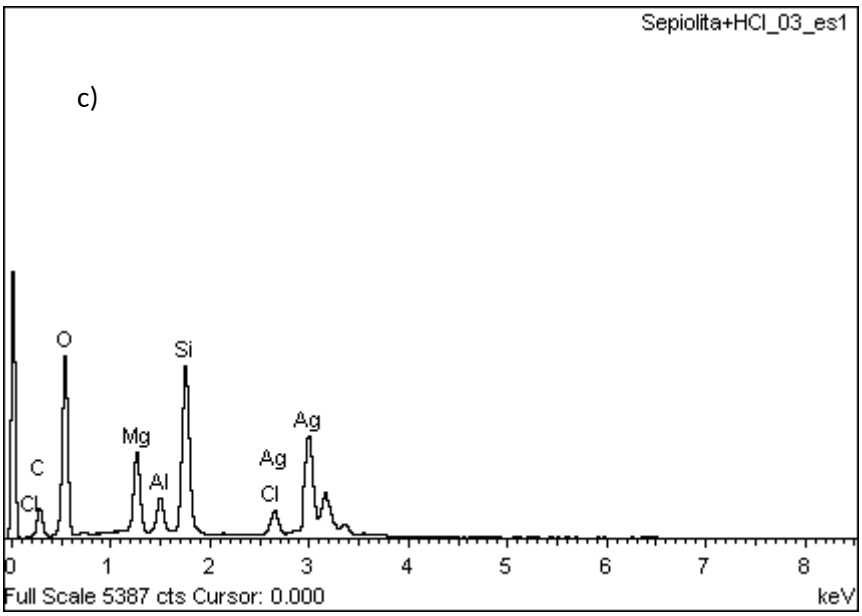
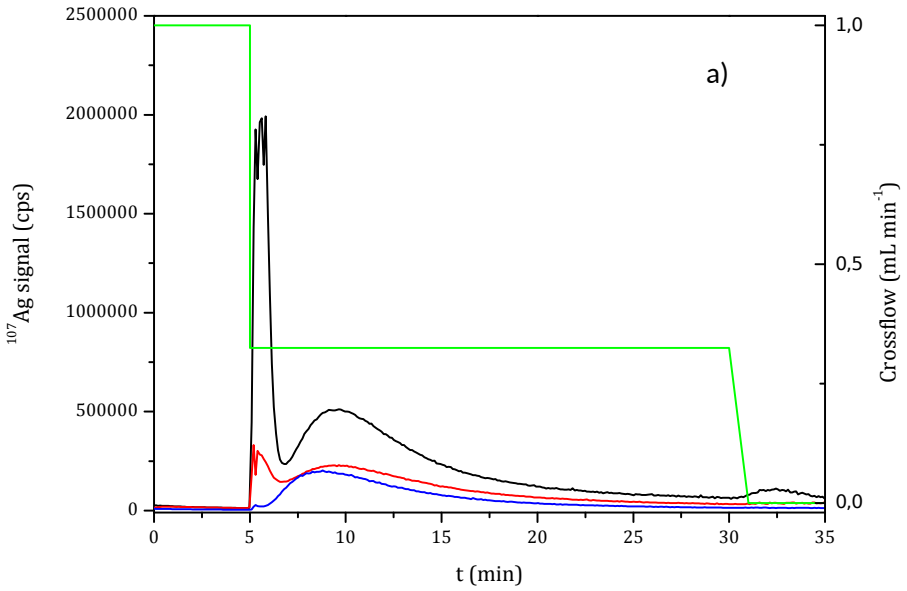


Figure S2 FESEM images. Microparticles of sepiolite-Ag a) with white spots on the surface (x1000) after treatment with HCl 0.01 M. b) Magnified micrograph from inset in a) (x85450). c) EDX spectra from white spots in a), point 1 in green.



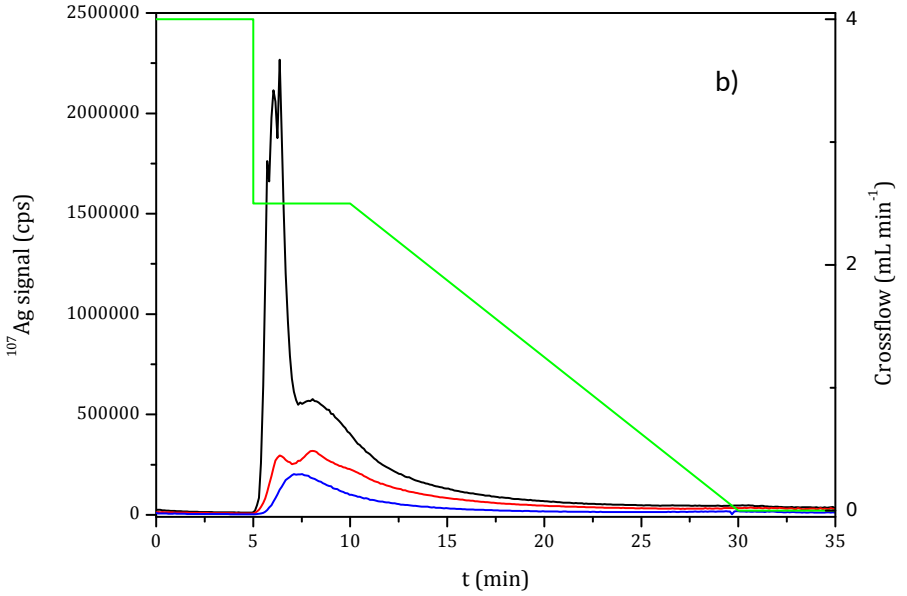


Figure S3 Comparison of the AF4-ICPMS fractograms of the suspensions from kaolin-Ag in ultrapure water (blue), from Step 2 (black) and Step 3 (red) from an *in vitro* digestion process using a) Crossflow program A (green line) and b) Crossflow program B (green line).