

Papain-mediated Synthesis of Silver Nanoparticles and Their Use in the Fluorescence-based Analysis of Folic Acid

Ma. Danica A. Magpantay, Maritess M. Lacquio, Mariam C. Recuenco*

Institute of Chemistry, University of the Philippines Los Baños, Brgy. Batong Malake, Los Baños, Laguna 4031

*Author to whom correspondence should be addressed; email: mrecuenco@up.edu.ph

ABSTRACT

Silver nanoparticles (AgNPs) were synthesized using an aqueous extract of papain as a reducing and a stabilizing agent for the fluorescence-based sensing of the B-vitamin, folic acid. The AgNPs obtained were spherical with diameters between 3-40 nm, with an average diameter of 11.4 ± 6.4 nm. The AgNPs also exhibited surface plasmon resonance (SPR) at 420 nm and fluorescence upon excitation with UV light. The addition of increments of folic acid caused the quenching of the AgNPs' fluorescence which could be fitted to the Stern-Volmer equation. The limits of detection (LOD) and quantification (LOQ) of the AgNPs as folic acid sensors were 1.10 $\mu\text{g/mL}$ and 3.31 $\mu\text{g/mL}$, respectively. Analysis of folic acid in selected over-the-counter vitamin and mineral supplements showed stark discrepancies between declared and experimental folic acid levels. Thus, it was inferred that other substances, such as metal ions and other vitamins, could interfere in folic acid analysis. While the use of papain-AgNPs as sensors seems promising, more work should be done for the improved selectivity and specificity for folic acid.

Keywords: *folic acid; fluorescence; papain; silver nanoparticles*

INTRODUCTION

Silver nanoparticles (AgNPs) are particles of silver with particle sizes between 1 to 100 nm. With their distinct optical, electrical, and thermal properties, AgNPs were being explored for their applications in catalysis, use as materials for solar cells, as agents for bioimaging and biosensing, and as materials for cosmetic and biomedical applications (Sarsar et al., 2014).

AgNPs can be prepared by the top-down and bottom-up approaches (Sarsar et al., 2014; Zhang et al., 2016). In the top-down approach, the bulk material is made smaller by breaking it up using processes like crushing, milling, or grinding. In the bottom-up approach, nanomaterials are built from atoms, molecules, or clusters. This approach is more often used due to the ability to generate

a uniform size, shape, and distribution for the material. Under the bottom-up approach are chemical methods and biological material-based methods utilizing microorganisms, plants, and biomolecules. The latter set of methods are considered to be green methods and are particularly attractive because of their economy, ease, safety, environment-friendliness, and potential biocompatibility for medical applications (Sarsar et al., 2014).

Plant extracts are among the most widely used materials used in the synthesis of metal nanoparticles. There has been an enormous increase in studies using various plant species and plant materials in the synthesis of metal nanoparticles, including AgNPs. Major biomolecules and secondary metabolites are regarded as essential in green synthesis, serving as reducing and stabilizing or capping agents for the metal nanoparticles (Kumar and Yadav, 2009).

Extracts from the papaya plant have been used in the synthesis of AgNPs (Banala et al., 2015; Singh et al., 2021). There were a number of reports of AgNPs exhibiting notable antibacterial properties (Rai et al., 2009), mild to moderate antifungal activities (Singh et al., 2024), and cytotoxicity against cancer cell lines (Shi et al., 2018). In addition, papaya extract-based synthesized AgNPs were shown to be capable of acting as sensors for heavy metals such as Fe (III) and Hg (II) (Revathi et al., 2023) and for organic and biological compounds such as tryptophan (Haridas et al., 2023) and paracetamol (Zamarchi and Cruz Vieira, 2021).

Among the components of the papaya extract, the protein papain may have roles in the reported synthesis of AgNPs. Papain is an enzyme, particularly a cysteine protease with a wide range of applications in the food and pharmaceutical, cosmetics, leather, and textile industries. Papain has a molecular weight of 23 kDa, an optimal temperature of 65 °C, and a wide pH range (5-8) for its activity (Singh et al., 2019). Papain is extracted from the latex of developing fruits. In the study of Monti et al. (2000), latex from 0.5 to 1.0 kg of papaya fruits could produce about 9 g latex per fruit, while papain from the latex could yield 1.51 mg to 2.43 mg per gram latex.

In the green synthesis of metal nanoparticles, proteins such as bovine serum albumin, trypsin, lysozyme and papain could serve as reducing agents to metal ions, mainly through the thiol groups of cysteine. The synthesis is aided by alkali, such as sodium hydroxide which removes protons from the cysteine and tyrosine side chains. The base may also act as a reductant by itself. Once reduced, the metal atoms form clusters leading to the formation of nanoparticles. Proteins stabilize the metal nanoparticles by surrounding them with negative charges through carboxyl, thiolate, and phenoxy groups, and complex formation with the nitrogen atoms of amino acids such as histidine, lysine, arginine, and tryptophan (Chen et al., 2013; Si and Mandal, 2007)).

In the study of Sangwan and Seth (2023), citrate-stabilized gold nanoparticles were functionalized with papain in an alkaline medium. These nanoparticles were tested as colorimetric sensors for mercury in milk. The study of Das et al. (2023) reported the use of papaya latex in the synthesis of prism-shaped gold nanozymes that were able to exhibit proteolytic activity. The study of Xu and Man (2021) reported the use of papain-mediated synthesized gold nanoparticles as a drug delivery system for the anti-androgen drug flutamide to improve the potency of the drug for prostate cancer.

Folic acid, the synthetic form of vitamin B9, is a crucial nutrient in human nutrition (Lucock, 2000). Its name comes from the Latin "folium," meaning leaf, reflecting its presence in green leafy vegetables (Lucock, 2000). This water-soluble vitamin is converted to 5-methyltetrahydrofolate in the human body. Folate is essential for cell metabolism and in the synthesis of nucleotides, which are components of the genetic material and enzyme cofactors. Deficiencies can lead to pernicious anemia in pregnant women and neural tube defects in embryos. Due to the link between folic acid and these disorders, many countries now require the fortification of wheat flour and certain cereals (Murphy and Westmark, 2020). Thus, it is important to develop reliable

methods for analyzing folic acid to ensure accurate measurements of the vitamin in food products, supplements, and in biological samples.

The microbiological assay, high-performance liquid chromatography (HPLC), and enzyme protein binding assay (EPBA) are the principal methods used for folic acid analysis (Arcot and Shrestha, 2005). While wet chemical analysis methods such as titrimetry, colorimetry, and spectrophotometry (using UV, visible, or infrared light) have been proposed, they are rarely utilized for routine folic acid analysis in food (Arcot and Shrestha, 2005). Recent studies have effectively demonstrated folic acid detection and quantification using silver and gold nanoparticles (Zhang et al., 2014; Hemmateenejad et al., 2014). Notably, Hemmateenejad et al. (2014) reported the use of bovine serum albumin as a stabilizing agent in the synthesis of gold nanoclusters for folic acid sensing. Furthermore, we have reported the synthesis of gold nanoparticles using salt-soluble wheat flour protein extract (Concepcion et al., 2022) and BSA-stabilized copper nanoclusters (Munar et al., 2024) for folic acid analysis via fluorescence quenching.

This study aimed to address the lack of routine wet chemical methods for folic acid analysis. We focused on utilizing green-synthesized silver nanoparticles (AgNPs) as sensors in a fluorescence-based spectrophotometric method for folic acid analysis. Initially, we describe the synthesis and characterization of silver nanoparticles produced using a papaya digestive aid supplement containing papain. Our sensing and quantification experiments demonstrate quenching of the AgNPs' fluorescence in response to increasing folic acid concentrations. By applying the proposed protocol to samples containing folic acid, we identified potential applications, limitations, and areas for improvement within this new method.

METHODS

Materials and Equipment. Healthy Options Chewable Natural Papaya Plus Enzyme Tablets with a serving size of 2 tablets (1.74 g) contain 200 mg papain and 20 mg enzyme blend. To prepare the papain solution, the tablets were weighed, powdered, and mixed with distilled water to dissolve the proteins. The mixture was centrifuged to separate the supernatant from the undissolved material. The folic acid used as standard was from Sigma-Aldrich (F7876). Folic acid solutions were freshly prepared by dissolution in 0.1 M NaOH. Transmission electron microscopy (TEM) of the AgNPs was performed using the JEOL JEM 1010 Electron Microscope at the Southeast Asian Fisheries Development Center (SEAFDEC) in Tigbauan, Iloilo. UV-Visible spectrophotometry measurements were performed using Thermo Scientific Multiskan GO UV-Vis microplate spectrophotometer (Thermo Fisher Scientific Inc., MA, USA). The fluorescence measurements were performed using Shimadzu RF-5301 Spectrofluorophotometer (Kyoto, Japan).

Papain-mediated synthesis of silver nanoparticles (AgNPs). The procedure for papain-mediated synthesis of AgNPs, designated as papain-AgNPs was based on the method used by Xie et al. (2009). Five mL of 50 mg/mL papain solution from the papaya tablets was mixed with 5 mL of 10 mM AgNO₃. The resulting solution was stirred vigorously in an enclosed vial for 2 minutes prior to the addition of 0.50 mL of 1 M NaOH. The mixture was continuously stirred for 24 hours at room temperature.

Characterization of the synthesized papain-AgNPs. The synthesized papain-AgNPs were subjected to transmission electron microscopy (TEM) to determine the size and shape of the nanoparticles. Sizes in diameter were measured from the TEM images using Image J. The UV-visible absorption spectrum of the nanoparticles was determined between the wavelength range 200-1000 nm. The fluorescence excitation and emission properties of the AgNPs were determined using a spectrofluorophotometer.

Determination of folic acid using the papain-AgNPs. The method to determine the effects of folic acid concentration on the fluorescence of papain-AgNPs was adapted from Hemmateenejad et al. (2014). Briefly, a 3-mL dilute solution of AgNP in water was added with incremental amounts of $1\mu\text{g}/\mu\text{L}$ folic acid in microliter quantities. The fluorescence intensities of the AgNPs were measured at 645 nm upon excitation at 320 nm. The results were fitted into the Stern-Volmer equation to obtain the limits of detection (LOD) and quantification (LOQ). The Stern-Volmer relationship is given by: $I_0/I = 1 + k [Q]$ where I_0 is the intensity without a quencher, I is the intensity with a quencher, k is the Stern-Volmer constant which is the product of the quenching rate constant and the radiative lifetime, and $[Q]$ is the concentration of the quencher. The LOD was calculated as $\text{LOD} = 3.3\sigma / k$, and the LOQ as $\text{LOQ} = 10\sigma / k$. The σ is the standard deviation of the calibration curve and k is the slope of the calibration curve and also the Stern-Volmer constant.

For the determination of folic acid in the vitamin and mineral preparations, the samples (2 tablets or capsules) were weighed and suspended in 0.1 M NaOH. The mixtures were either centrifuged or filtered to obtain the clarified supernatant or filtrate. The sample and standard folic acid solutions were added in incremental amounts to AgNP solutions as described above. The ratios of initial to final fluorescence intensities and volumes added were plotted to obtain linear plots. To determine the sample's folic acid concentration, the slope from the plot was compared with the slope from the standard folic acid plot for $1\mu\text{g}/\mu\text{L}$ folic acid. Dilutions and weights of the samples were noted and then later used in the calculations of the folic acid content in the vitamin and mineral preparations.

Effects of metal ions on the fluorescence of papain-AgNPs. Aqueous solutions (5 mM) of metal ions such as aluminum (III), cadmium (II), cobalt (II), chromium (II), copper (II), iron (III), mercury (II), magnesium (II), manganese (II), nickel (II), lead (II), and zinc (III) were prepared from their corresponding salts. The solutions of metal ions were added to the synthesized AgNPs (final concentration of 10 μM) to observe their effects on the fluorescence intensities of the AgNPs.

RESULTS AND DISCUSSION

Synthesis of papain-AgNPs. A color change from colorless to yellowish brown was observed upon the addition of 1M NaOH to the colorless AgNO_3 -papain protein solution, indicating the formation of AgNPs. After 24 hours of stirring, the brown-colored mixture obtained was designated as the papain-AgNPs (Figure 1A Inset). The mechanism of AgNP synthesis may be hypothesized as involving the reduction of Ag^+ to Ag^0 with the aid of the proteins' cysteine, histidine, tryptophan, and tyrosine residues (Chen et al., 2013). The addition of 1 M NaOH likely aided in the denaturation of the protein as well as in the dissociation of disulfide bonds (Sathishkumar et al., 2009). The basic pH also promotes deprotonation of the carboxyl groups, phenolic groups of tyrosine and thiol groups of cysteine, making the protein a better complexing and stabilizing agent. The nanoparticles obtained may be composed of a metallic core of Ag^0 clusters coated with a layer of the Ag(I) -thiolate-protein (Battocchio et al., 2012), with the protein in its random coil form (El-Sayed and Schneider, 2020).

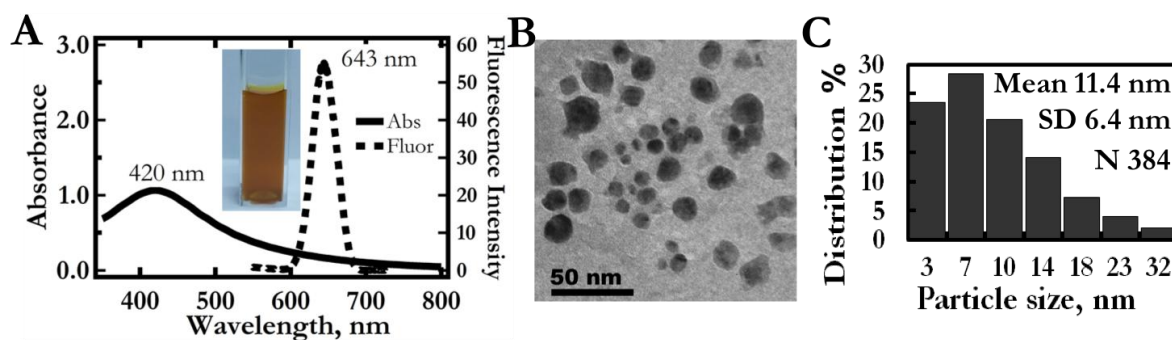


Figure 1. Optical and physical properties of the synthesized papain-AgNPs. A. The UV-visible absorption (solid line) of the AgNPs in solution (inset) shows a surface plasmon resonance peak at 420 nm. The fluorescence emission spectra (dashed line) show a fluorescence emission peak at 643 nm upon excitation at 320 nm; B. Transmission electron microscopy image shows spherical AgNPs; C. Histogram of the particle size distribution shows that the synthesized AgNPs vary in sizes with an average diameter of $11.4 \text{ nm} \pm 6.4 \text{ nm}$.

Characterization of papain-AgNPs. The synthesized AgNPs exhibited an absorption spectrum with a peak at 420 nm when subjected to UV-visible spectrophotometry (Figure 1 A, solid line). This absorption peak corresponds to the surface plasmon resonance (SPR) property of silver nanoparticles. The SPR of metal nanoparticles is due to the oscillation of conduction electrons upon light irradiation. The SPR peak observed agrees with those of AgNPs synthesized by citrate reduction (Mahmudin et al., 2024) and by green synthesis (Lu et al., 2012). In addition, the observed spectrum could indicate spherical AgNPs (Amendola et al., 2010).

At an excitation wavelength of 320 nm, the synthesized AgNPs exhibited fluorescence emission with a peak at 643 nm (Figure 1A, dashed line). The spectrum agreed with previous reports (Ashenfelter et al., 2015). While fluorescence of metal nanoparticles was believed to be size-dependent and could originate from the quantum states within the metal core or mixed ligand states, Ashenfelter et al. (2015) reported that fluorescence could be attributed to states related to the ligand-metal interface. Furthermore, smaller metal nanoparticles such as nanoclusters were reported to have no surface plasmon resonance but exhibit fluorescence emission when irradiated by UV light (Xie et al., 2009). The synthesized AgNPs in this study both exhibited SPR and fluorescence. This may indicate the presence of both the larger nanoparticles and the tiny nanoclusters (Zhu et al., 2020).

The synthesized AgNPs were shown to be generally spherical in shape and with sizes of $11.4 \pm 6.4 \text{ nm}$ (Figure 1B). The microscopy result confirms the variations in the sizes of the AgNPs. Based on the histogram in Figure 1C, a significant number of smaller-sized nanoparticles are about 3-5 nm in size. There may also be particles much smaller than these and may include some nanoclusters. These could account for the particles that exhibited fluorescence at 640 nm upon excitation at 320 nm. Zhu et al. (2020) reported the synthesis of fluorescent silver nanoclusters from plasmonic nanoparticles by the top-down approach via a ligand-induced etching process. Silver nanoclusters with sizes less than 2 nm could exhibit intense photoluminescence (Zhu et al., 2020).

Interaction of folic acid and papain-AgNPs. The addition of incremental amounts of folic acid to a diluted solution of AgNPs decreased or quenched the fluorescence of the nanoparticles (Figure 2A). There are a variety of different mechanisms that result in the quenching of fluorescence. These include excited state reactions, energy transfer, complex formation, and collisional quenching (Albani, 2004). One of these possibilities includes the electron transfer from the excited state of fluorescent nanoparticles to the quencher. The close proximity of a substance such as folic acid to the fluorescent metal nanoparticles allows the energy transfer from the

nanoparticles. There is also the possibility of complex formation between the fluorescent material and the quencher is referred to as static quenching. Folic acid could be acting as ligands to the Ag atoms on the surface of the nanoparticles. In collisional quenching, the excited state of the fluorescent material is lost by contact with the quencher. In this study, we can only speculate that the quenching observed might be a combination of the above-mentioned mechanisms.

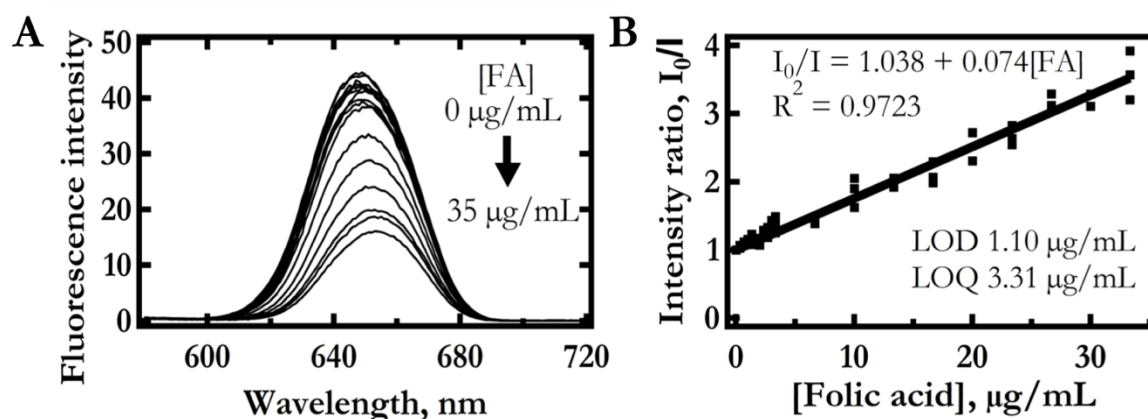


Figure 2. Effect of folic acid (FA) on the fluorescence of the papain-AgNPs. A. The fluorescence emission peak at 643 nm of the AgNPs decreases incrementally upon microliter addition of 1 µg/µl FA. The final concentration of FA in the solution is indicated. The excitation wavelength was 320 nm. B. Plot of folic acid concentration µg/µl versus fluorescence intensity ratios (I_0/I) where I_0 and I are initial and final intensities, respectively. The plot was fitted to the Stern-Volmer model of quenching and the equation and R^2 are indicated. From the regression data, the limits of detection and quantification were determined as 1.10 µg/mL and 3.31 µg/mL, respectively.

Papain-AgNPs as sensors for folic acid. By quantifying the decrease in fluorescence upon the addition of folic acid to the AgNPs (Figure 2A), we were able to fit the data to the Stern-Volmer relationship, which allows the kinetics of fluorescence quenching to be explored (Figure 2B). The data showed the linear relationship between the amount of folic acid added and the ratios of initial to final fluorescence intensities of the AgNPs (Figure 3). From the linear equation, the calculated limit of detection (LOD) was 1.10 µg/mL, while the limit of quantitation (LOQ) was 3.31 µg/mL.

Table 1 shows the figures of merit—linear range, LOD, and LOQ of several fluorescence-based folic acid analyses using protein-stabilized metal nanomaterial. The LOD and LOQ of this study are comparable with our previous studies (Concepcion et al., 2022; Munar et al., 2024). In terms of working linear range, this study with 3.31–35.00 µg/mL has a wider linear range compared to the studies of Concepcion et al. (2022) and Munar et al. (2024). These three studies, having been conducted in the same environment, could be observed to have relative closeness in terms of the figures of merit. Factors such as temperature, humidity, instrument conditions, reagents, and even the level of expertise of the researchers may have some influence on these studies. Conversely, it seems apparent that the previous studies (Hemmateenejad et al., 2014; Fereja et al., 2021; Li et al., 2019) had much lower LOD values in the ng/mL levels compared to this study. In addition, the linear ranges of these studies have a wider range starting from the ng/mL levels until the µg/mL levels. It seems that these previous studies may have better applicability in terms of the amounts of folic acid that could be analyzed. Better figures of merit and a wider linear range may be achieved by conducting more experiments to finetune the techniques and conditions in the analysis.

Table 1. Limits of detection and quantification of fluorescence-based methods for folic acid analysis using protein-stabilized metal nanoparticles.

Nanomaterial	Linear Range	Limit of detection	Limit of quantification	Reference
Papain-silver nanoparticles	3.31-35.00 µg/mL	1.10 µg/mL	3.31 µg/mL	This study
Bovine serum albumin (BSA)-copper nanoclusters	2.35-6.00 µg/mL	0.78 µg/mL	2.35 µg/mL	Munar et al., 2024
Wheat flour salt soluble proteins-gold nanoparticles	3.10-22.00 µg/mL	0.92 µg/mL	3.10 µg/mL	Concepcion et al., 2022
BSA-gold nanoclusters	120 ng/mL-33.12 µg/mL	18.3 ng/mL	120 ng/mL	Hemmateenejad et al., 2014
BSA-capped Au-Ag bimetallic nanoclusters	0-100 µM (0-44.14 µg/mL)	0.47 nM (0.21 ng/mL)		Fereja et al., 2021
Ovalbumin-stabilized copper nanoclusters	0.2-200 µM (0.88-88.28 µg/mL)	0.18 µM (79.4 ng/mL)		Li et al., 2019

Fluorescence quenching-based quantification of folic acid from commercial supplements.

The results of detection and quantification of folic acid through fluorescence quenching of three over-the-counter pharmaceutical preparations containing folic acid, and a non-folic acid analgesic drug are shown in Table 2.

Among the samples tested, only the experimental content of the folic acid supplement came close to the manufacturer's claim. This sample contains only folic acid as an active ingredient. The iron supplement and the multivitamin samples contain other components. The iron supplement contains Mn, Cu, ascorbic acid, and cyanocobalamin in addition to folic acid. The multivitamin sample contains a total of 26 components including 15 vitamins and 11 minerals. In the multivitamin and iron supplement, the experimental contents of folic acid determined were way too large by 500-800%. The very large discrepancy compared to the manufacturer's claim could be due to the interactions of other components with the AgNPs. This suggests that the current method suffers from influences by interfering substances.

Table 2. Fluorescence-based analysis of folic acid content of selected samples using papain-AgNPs.

	R ²	Slope (I ₀ /I/volume added) ¹	FA Concentration ² (µg/µL)	Total Volume (mL)	Total FA in 2 tablets or capsules (µg)	FA Content Experiment (µg/tablet or capsule)	FA Content Manufacturer's Claim (µg)	% Error ³
Folic acid standard (1µg/µL)	0.9673	0.0236	1.000	-	-	-	-	-
Samples for FA analysis								
Folic acid Supplement	0.9289	0.0275	1.165	10	11653	5826	5000	17
Iron Supplement	0.9535	0.0447	1.894	10	18941	9470	1000	847
Multivitamins	0.6955	0.0116	0.492	10	4915	2458	400	514
Analgesic	-0.451	(-0.0013)	n.d.	10	n.d.	n.d.	0	n.d.

Information about the samples' composition (per one tablet or capsule)

Folic acid supplement: folic acid 5 mg

Iron supplement: ferrous gluconate 250 mg, manganese sulfate 0.2 mg, copper sulfate 0.2 mg, vitamin C 50 mg, folic acid 1 mg, vitamin B12 7.5 mcg, sorbitol 25 mg

Multivitamins: Vitamins A, E, C, K, B1, B2, B6, B12, D, biotin, folic acid 400 mcg, niacin, pantothenic acid, calcium, phosphorus, magnesium, iron, iodine, copper, manganese, chromium, molybdenum, selenium, zinc

Analgesic: Acetaminophen 500 mg

¹Soluble components were extracted from two tablets or capsules using 10 mL of 0.1N NaOH as the solvent. Undissolved residues were removed, and the resulting solutions were utilized to titrate 3 mL of papain-AgNPs. The fluorescence intensity ratios were then plotted against the volumes of the sample added to the AgNPs.

²FA concentration was determined against the slope of the folic acid standard at 1 $\mu\text{g}/\mu\text{L}$.

³%Error = $[\text{FA experiment} - \text{FA manufacturer's claim} / \text{FA manufacturer's claim}] \times 100\%$

In the case of the analgesic, the plots were non-linear, and the fitted line was nearly flat and fitted with a negative slope (Figure 3). This may imply that the proposed method may have the potential to distinguish between the analyte and other substances. This is supported by the observation that the folic acid standard and the folic acid supplement almost have the same slope. This could be an indication that the proposed method may work for samples mainly composed of folic acid. For the multivitamin sample, the plot showed a weakly positive slope and lower R-squared (Table 2, Figure 3), an indication of a weak fit of the response to the folic acid in the sample. This may be due to the influence of the other components interfering with the analysis. On the other hand, for the iron supplement, the R-squared is high, an indication of a good fit (Table 2, Figure 3). However, the slope is about twice the folic acid standard. The iron supplement per tablet has 250 mg ferrous gluconate as the main component compared to the 1 mg folic acid content. This observed behavior is in agreement with the observations of Hemmateenejad et al. (2014) that Fe interferes with folic acid analysis at higher concentration ratios greater than 5.

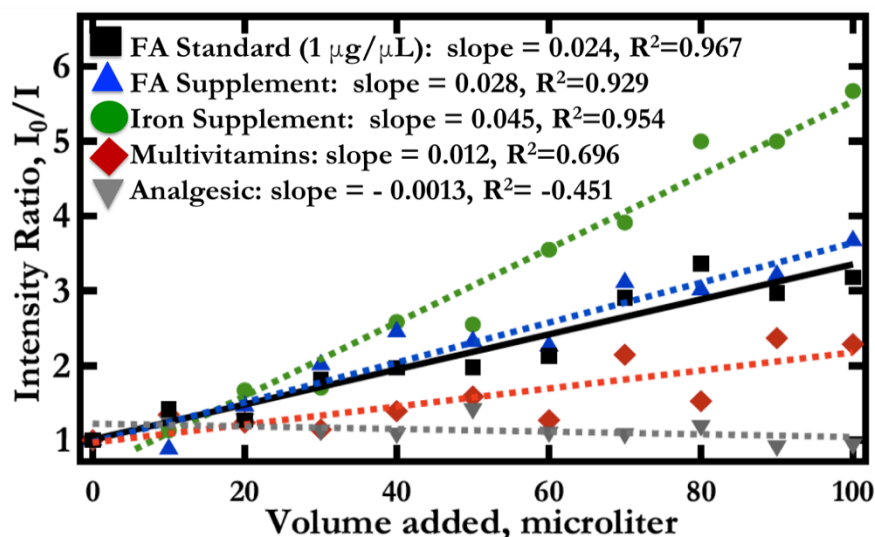


Figure 3. Plot of the fluorescence intensity ratio (I_0/I) against volume of added folic acid (FA) standard (1 $\mu\text{g}/\mu\text{L}$), and plots of (I_0/I) against volume of added solutions of samples. I_0 is the initial intensity and I is the final intensity. Regression analysis was performed to determine the slopes and R-squared. Soluble components were extracted from two tablets or capsules using 10 mL of 0.1N NaOH as the solvent. Undissolved residues were removed, and the resulting solutions were utilized to titrate 3 mL of papain-AgNPs. The fluorescence intensity ratios were then plotted against the volumes of the sample added to the AgNPs.

The percent errors obtained in this study were relatively high for the folic acid supplement and very high for the iron supplement and multivitamins. We also had some relatively large percent errors in the folic acid contents compared to the declared values in our previous studies (Concepcion et al., 2022; Munar et al., 2024). In contrast, other published studies (Hemmateenejad et al., 2014; Fereja et al., 2021; Li et al., 2019) had much better % recoveries nearing 100% and seemingly better reliability. It is therefore important to determine the specific conditions in sample preparations, in the nanoparticle preparations, in the instrument settings and in the other steps that could minimize the effects of interfering substances.

Effect of metals ions on the fluorescence of papain-AgNPs. This study also looked into the possible effects of metal ions on the emission spectra of the synthesized AgNPs (Figure 4). The 10 metal ions tested were aluminum, cadmium, cobalt, chromium, copper, iron, mercury, magnesium, manganese, nickel, lead, and zinc. All, except for chromium, enhanced the fluorescence of the papain-AgNPs (Figure 4). The results suggest that the fluorescence of the synthesized AgNPs is affected by metal ions and is not completely selective towards folic acid.

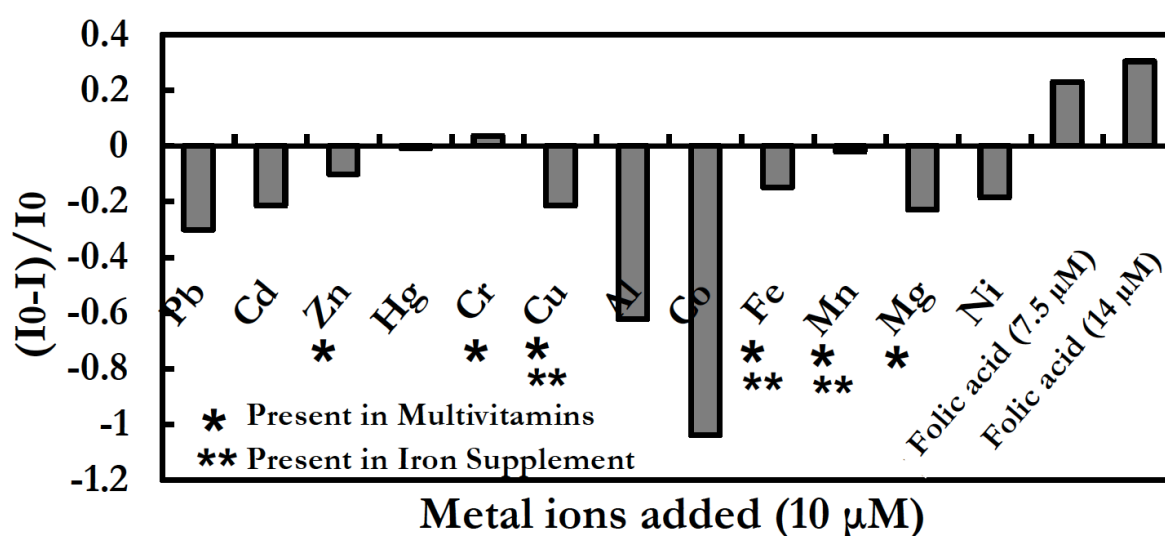


Figure 4. Effects of addition of some metal ions (10 μM final concentration) on the fluorescence emission (at 643 nm) of papain-AgNPs. The $(I_0-I)/I_0$ on the y-axis represents the fraction of fluorescence enhancement (negative values) or quenching (positive values) in the presence of metals or folic acid. I_0 is the initial intensity and I is the final intensity. All the metal ions added, except Cr, enhanced the fluorescence of the AgNPs. Asterisks indicated the metal ions that were present in the multivitamins and iron supplement for folic acid content analysis.

The added metal ions may become reduced in the environment of the papain-AgNPs and become fluorescent themselves thus adding to the fluorescence detected. These fluorescent molecules that interact with the AgNPs surface could have coupling between the electromagnetic field and spatially confined free-electrons leading to higher emission intensities (Jeong et al., 2018).

These present results are in contrast with the reported quenching of AgNP fluorescence by metal ions Cd, Zn, Pb, Co, Cu, Ba, Mn, Hg, Ca, Mg, Sr and Ni. (Makwana et al., 2016). Hemmateenejad et al. (2014) reported that in the analysis of folic acid using BSA-stabilized gold nanoclusters, the metal ions Ca, Zn, Al, Pb, and Cd, showed no interference. Meanwhile, Fe was reported to interfere at higher concentration ratios. Moreover, an equal amount of Ag, Cu, and Ni ions may interfere with the determination of folic acid in their method (Hemmateenejad et al. (2014).

Hemmateenejad et al. (2014) suggested that using EDTA as a chelating agent may possibly reduce the interference of metal ions in the analysis of folic acid. However, in our experience, EDTA may also interfere with the analysis. Hence, the more viable solution may be to extract folic acid from the sample using techniques such as thin-layer chromatography, column chromatography, or solid-liquid or liquid-liquid extraction.

CONCLUSIONS

This study demonstrated the green synthesis of silver nanoparticles (AgNPs) utilizing a commercially available papaya supplement, primarily composed of papain, which acted as both the stabilizing and reducing agent. The AgNPs exhibited characteristic optical properties, including surface plasmon resonance and fluorescence when excited by UV light. As a sensor for the quantitative analysis of folic acid, the AgNPs show potential, provided that the samples are free from interfering substances such as heavy metal ions. Therefore, future research should focus on enhancing specificity and sensitivity, as well as establishing an efficient method for processing folic acid-containing samples.

ACKNOWLEDGEMENT

This work was supported by the UPLB Basic Research Program (Project ID 17855).

REFERENCES

- Albani JR. Structure and dynamics of macromolecules: absorption and fluorescence studies. 1st ed. The Netherlands: Elsevier Science; 2004.
- Amendola V, Bakr OM, Stellacci F. A study of the surface plasmon resonance of silver nanoparticles by the discrete dipole approximation method: effect of shape, size, structure, and assembly. *Plasmonics*. 2010 Jan; 5: 85–97. <http://dx.doi.org/10.1007/s11468-009-9120-4>
- Arcot J, Shrestha A. Folate: methods of analysis. *Trends Food Sci Technol*. 2005 Jun; 16(6-7)253-266. <https://doi.org/10.1016/j.tifs.2005.03.013>
- Ashenfelter BA, Desiredy A, Yau SH, Goodson T, Bigioni TP. Fluorescence from molecular silver nanoparticles. *J Phys Chem C*. 2015 Aug; 119(35):20728-20734. <https://doi.org/10.1021/acs.jpcc.5b05735>
- Banala RR, Nagati VB, Karnati PR. Green synthesis and characterization of *Carica papaya* leaf extract coated silver nanoparticles through X-ray diffraction, electron microscopy and evaluation of bactericidal properties. *Saudi J Biol Sci* 2015 Sep; 22(5):637-644. <https://doi.org/10.1016/j.sjbs.2015.01.007>
- Battocchio C, Meneghini C, Fratoddi I, Venditti I, Russo MV, Aquilanti G, et al. Silver nanoparticles stabilized with thiols: a close look at the local chemistry and chemical structure. *J Phys Chem C*. 2012 Aug; 116(36):19571–19578. <https://doi.org/10.1021/jp305748a>
- Chen Y, Wang Y, Wang C, Li W, Zhou H, Jiao H, Lin Q, and Yu C. Papain-directed synthesis of luminescent gold nanoclusters and the sensitive detection of Cu²⁺. *J Colloid Interface Sci*. 2013 Apr; 396:63-68. <https://doi.org/10.1016/j.jcis.2013.01.031>

Concepcion CA, Lacquio M, Peralta M, Recuenco M. Synthesis of gold nanoparticles (AuNPs) using salt-soluble wheat flour proteins and their use in the fluorescence-based sensing of folic acid. KIMIKA. 2022 Dec; 33(2):44–58. <https://doi.org/10.26534/kimika.v33i2.44-58>

Das AK, Kalita JJ, Borah M, Das S, Sharma M, Saharia D, et al. Papaya latex mediated synthesis of prism shaped proteolytic gold nanozymes. Sci Rep. 2023 Apr; 13:5965. <https://doi.org/10.1038/s41598-023-32409-7>

El-Sayed N., Schneider M. Advances in biomedical and pharmaceutical applications of protein-stabilized gold nanoclusters. 2020 Aug; J Mater Chem B. 8:8952-8971. <https://doi.org/10.1039/D0TB01610A>

Fereja SL, Li P, Guo J, Fang Z, Zhang Z, Zhuang Z, et al. Silver-enhanced fluorescence of bimetallic Au/Ag nanoclusters as ultrasensitive sensing probe for the detection of folic acid. Talanta. 2021 Oct; 233:122469. <https://doi.org/10.1016/j.talanta.2021.122469>

Haridas ES, Bhattacharya S, Varma MKR, Chandra GK. *Carica papaya* male flower mediated environmentally benign synthesis of silver nanoparticles towards the sensing of tryptophan. Mater. Today: Proc. 2023. ISSN 2214-7853. In press. <https://doi.org/10.1016/j.matpr.2023.03.731>

Hemmateenejad B, Shakerizadeh-Shirazi F, Samari F. BSA-modified gold nanoclusters for sensing of folic acid. Sens. Actuators B Chem. 2014 Aug; 199:42–46. <https://doi.org/10.1016/j.snb.2014.03.075>

Jeong Y, Kook YM, Lee K, Koh WG. Metal enhanced fluorescence (MEF) for biosensors: general approaches and a review of recent developments. Biosens Bioelectron. 2018 Jul; 111:102–116. <https://doi.org/10.1016/j.bios.2018.04.007>

Kumar V, Yadav SK. Plant-mediated synthesis of silver and gold nanoparticles and their applications. J Chem Technol Biotechnol. 2009 Feb; 84(2):151–157. <https://doi.org/10.1002/jctb.2023>

Li X, Wu X, Zhang F, Zhao B, Li Y. Label-free detection of folic acid using a sensitive fluorescent probe based on ovalbumin stabilized copper nanoclusters. Talanta. 2019 Apr; 195:372–380. <https://doi.org/10.1016/j.talanta.2018.11.067>

Lucock M. Folic acid: nutritional biochemistry, molecular biology, and role in disease processes. Mol Genet Metab. 2000 Sep-Oct; 71(1-2):121–138. <https://doi.org/10.1006/mgme.2000.3027>

Lu R, Yang D, Cui D, Wang Z, Guo L. Egg white-mediated green synthesis of silver nanoparticles with excellent biocompatibility and enhanced radiation effects on cancer cells. Int J Nanomedicine. 2012 Apr; 7:2101–7. <https://doi.org/10.2147%2FIJN.S29762>

Makwana B A, Vyas DJ, Bhatt KD, Darji S, Jain VK. Novel fluorescent silver nanoparticles: sensitive and selective turn off sensor for cadmium ions. Appl Nanosci. 2016 Apr; 6:555–566. <https://doi.org/10.1007/s13204-015-0459-x>

Mahmudin L, Wulandani R, Riswan M, Kurnia Sari E, Dwi Jayanti P, Syahrul Ulum M, et al. Silver nanoparticles-based localized surface plasmon resonance biosensor for Escherichia coli detection. Spectrochim Acta A Mol Biomol Spectrosc. 2024 Apr; 311:123985. <https://doi.org/10.1016/j.saa.2024.123985>

Monti R, Basilio CA, Trevisan HC, Contiero J. Purification of papain from fresh latex of *Carica papaya*. Braz Arch Biol Technol. 2000 43(5):501-507. <https://doi.org/10.1590/S1516-89132000000500009>

Munar KI, Lacquio M, Remillion BC, Recuenco M. Synthesis of BSA-stabilized copper nanoclusters and evaluation of their potential use as fluorescent sensors for folic acid. KIMIKA. 2024; 35(1):45-61. <https://doi.org/10.26534/kimika.v35i1.45-61>

Murphy ME, Westmark CJ. Folic acid fortification and neural tube defect risk: analysis of the food fortification initiative dataset. Nutrients. 2020 Jan; 12(1):247. <https://doi.org/10.3390%2Fnu12010247>

Rai M, Yadav A, Gade A. Silver nanoparticles as a new generation of antimicrobials. Biotechnol Adv. 2009 Jan-Feb; 27(1):76-83. <https://doi.org/10.1016/j.biotechadv.2008.09.002>

Revathi E, Yaku G, Unnisa SA, Malyala P, Praveen V. Microwave assisted green synthesis of silver nanoparticles from *Carica papaya* fruit extract: Characterization and detection of Fe³⁺ and Hg²⁺ ions. Mater Today: Proc. 2023 Apr. 92(2):490-497. <https://doi.org/10.1016/j.matpr.2023.03.597>

Sangwan S, Seth R. Synthesis and stability analysis of papain-functionalized gold nanoparticles (P-AuNPs) for the colorimetric detection of mercury in milk. Int J Dairy Technol. 2023 Jan; 76:351-363. <https://doi.org/10.1111/1471-0307.12936>

Sarsar V, Selwal KK, Selwal MK. Nanosilver: Potent antimicrobial agent and its biosynthesis. Afr J Biotechnol. 2014 Jan; 13(4):546-544. <https://doi.org/10.5897/AJB2013.13147>

Sathishkumar M, Sneha K, Won SW, Cho CW, Kim S, Yun YS. *Cinnamon zeylanicum* bark extract and powder mediated green synthesis of nano-crystalline silver particles and its bactericidal activity. Colloids Surf B Biointerfaces. 2009 Oct; 73(2):332-338. <https://doi.org/10.1016/j.colsurfb.2009.06.005>

Shi T, Sun X, He QY. Cytotoxicity of silver nanoparticles against bacteria and tumor cells. Curr Protein Pept Sci. 2018; 19(6):525-536. <https://doi.org/10.2174/1389203718666161108092149>

Si S, Mandal TK. Tryptophan-based peptides to synthesize gold and silver nanoparticles: a mechanistic and kinetic study. Chem Eur J. 2007 Mar; 13(11):3160-3168. <https://doi.org/10.1002/chem.200601492>

Singh PK, Shrivastava N, Ojha BK. Enzymes in the meat industry. In: Kuddus M, editor. Enzymes in food biotechnology. Academic Press; 2019. p. 111-128. <https://doi.org/10.1016/B978-0-12-813280-7.00008-6>

Singh SP, Mishra A, Shyanti RK, Singh RP, Acharya A. silver nanoparticles synthesized using *Carica papaya* leaf extract (AgNPs-PLE) causes cell cycle arrest and apoptosis in human prostate (du145) cancer cells. Biol Trace Elem Res. 2021 Apr; 199(4):1316-1331. <https://doi.org/10.1007/s12011-020-02255-z>

Singh J, Kumar A, Nayal AS, Vikal S, Shukla G, Singh A, et al. Comprehensive antifungal investigation of green synthesized silver nanoformulation against four agriculturally significant fungi and its cytotoxic applications. Sci Rep. 2024 Mar; 14:5934. <https://doi.org/10.1038/s41598-024-56619-9>

Xie J, Zheng Y, Ying JY. Protein-directed synthesis of highly fluorescent gold nanoclusters. J Am Chem Soc. 2009 Jan; 131(3):888-889. <https://doi.org/10.1021/ja806804u>

Xu X, Man L. Papain mediated synthesized gold nanoparticles enhance the potency of bioconjugated flutamide. *Curr Pharm Biotechnol.* 2021; 22(4):557-568.
<http://dx.doi.org/10.2174/1389201021666200227121144>

Zamarchi F, Vieira IC, Determination of paracetamol using a sensor based on green synthesis of silver nanoparticles in plant extract. *J Pharm Biomed Anal.* 2021 Mar; 196:113912.
<https://doi.org/10.1016/j.jpba.2021.113912>

Zhang JR, Wang ZL, Qu F, Luo HQ, Li NB. Polyethylenimine-capped silver nanoclusters as a fluorescence probe for highly sensitive detection of folic acid through a two-step electron-transfer process. *J Agric Food Chem.* 2014 Jun; 62(28):6592-6599.
<https://doi.org/10.1021/jf4054534>

Zhang XF, Liu ZG, Shen W, Gurunathan S. Silver nanoparticles: synthesis, characterization, properties, applications, and therapeutic approaches. *Int J Mol Sci.* 2016 Sep; 17(9):1534.
<https://doi.org/10.3390/ijms17091534>

Zhu L, Gharib M, Becker C, Zeng Y, Ziefuß AR, Chen L, et al. Synthesis of fluorescent silver nanoclusters: introducing bottom-up and top-down approaches to nanochemistry in a single laboratory class. *J Chem Educ.* 2020 Jan; 97(1):239-243.
<https://doi.org/10.1021/acs.jchemed.9b00342>