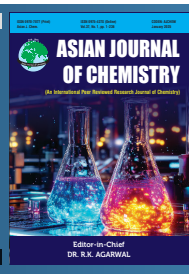
Asian Journal of Chemistry; Vol. 37, No. 5 (2025), 1183-1190

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2025.33466



Biochemical and Rapid Paper Sensory Detection of Heavy Metals in Milk Based on Biosynthesized Silver Nanoparticles

MIA TOM, SHEETHAL PRADEEP and MAJESH TOMSON*

Department of Life Siences, School of Sciences, Christ University, Bengaluru-560029, India

*Corresponding author: E-mail: majesh.tomson@christuniversity.in

Received: 2 February 2025; *Accepted*: 14 April 2025; *Published online*: 30 April 2025; AJC-21986

Milk is an emulsion of proteins and fats in water that contributes to a nutritious diet and enhances our immune system. However, contamination of heavy metals in milk due to an increase in industrialization and urbanization can be a serious threat to human health. This study focused on the rapid detection of heavy metals particularly lead and mercury in milk using biochemical assays as well as paper-based colorimetric sensor based on green synthesized silver nanoparticles (AgNPs) from leaf extract of *Hemigraphis colorata*. Biochemical assays such as the lead chromate test and sodium hydroxide test were employed to detect lead and mercury in milk samples. The biogenic AgNPs were characterized by UV–Vis spectroscopy, scanning electron microscope, Fourier transform infrared spectroscopy, energy dispersive X-ray analysis (EDX) and X-ray diffraction. The unique properties of silver nanoparticles (AgNPs) like surface plasma resonance (SPR), large surface area and visible colour change upon aggregation when metal ions interact, enable them to detect heavy metals. This is a portable and affordable method of detection that ensures safer milk consumption and sustainable environmental practices.

Keywords: Milk, Silver nanoparticles, Colorimetric sensor, Green synthesis, Heavy metals.

INTRODUCTION

Milk contains both macro- and micronutrients that are essential for immune system function, bone formation and growth and it also contains vitamins and unique fatty acids [1,2]. In its purest form, milk is an emulsion of protein and fat in water, along with dissolved minerals, vitamins and carbohydrates [3]. As the concentration of hazardous substances and environmental contaminants rises, milk and milk products become less safe. The increasing industrialization and urbanization have increased the accumulation of xenobiotics, particularly heavy metals. Its pollution has been a major health risk to humans, especially if it enters the body through the food chain [4]. Several diseases have been connected to heavy metal toxicity, but the severity of the condition increases significantly if the heavy metals are detected in milk, which is the staple diet for the vulnerable age group of individuals [5].

The different types of nanoparticles encompass organic, inorganic and carbon-based types and possess unique chemical, electrical and biological properties, making them suitable for various applications [6]. Noble metal nanoparticles, like silver nanoparticles (AgNPs), are increasingly studied due to their unique characteristics, such as chemical inertness, strong absorption of electromagnetic waves, biological compatibility and surface plasmon resonance. These properties make AgNPs valuable in diverse applications, including water purification [7], DNA sequencing [8], biological sensors [9], catalysis [10] and antimicrobial activities [11]. An eco-friendly, cost-effective and safer method for synthesizing AgNPs is essential due to their widespread use. In the past decade, green synthesis has gained recognition for using naturally accessible biological sources to obtain metallic nanoparticles [12]. This method is easy to use, low-maintenance, low-energy consumption and environmental friendly. In this work, leaves of *Hemigraphis colorata* are used for the green synthesis of AgNPs. This plant belongs to the Acanthaceae family and has therapeutic benefits for treatment of inflammation, bleeding, ulcers and wounds [13].

The colorimetric detection of heavy metals using green-synthesized nanoparticles has gained traction due to its simplicity, eco-friendliness and user-centric approach. This process typically operates *via* two distinct mechanisms. In the

first mechanism, a redox reaction occurs between the nanoparticles and the heavy metal, leading to the oxidation of the nanoparticles, which results in a visible colour change along with a blue shift in the UV-vis spectra, indicating a reduction in the nanoparticle peaks. In the second mechanism, heavy metals induce the aggregation or complexation of nanoparticles, resulting an increase in nanoparticle size. This aggregation is reflected as a redshift in the UV-vis spectra and is accompanied by a visible colour change [14]. Green-synthesized AgNPs offer eco-friendly, sensitive detection of heavy metals like Pb^{2+}, Hg^{2+}, Cd^{2+}, Ni^{2+}, Al^{3+} and Cu^{2+} through colour changes or LSPR shifts. The Pb^{2+} and Hg^{2+} were detected through the aggregation or oxidation-reduction, while Cd^{2+} and Ni^{2+} cause nanoparticle aggregation with visible colour changes. *Mentha arvensis* based AgNPs detect Al^{3+} with a yellow to reddish-brown shift and *Moringa oleifera* derived AgNPs iden-tify Cu^{2+} through brown-to-grey colour changes. These cost-effective methods are highly selective and applicable to environmental and biological samples [15].

There is a growing demand for affordable and rapid detection methods for diagnostics and environmental monitoring, particularly in developing regions. Paper-based devices, such as strip tests and microfluidics, are cost-effective, portable and easy to use, relying on capillary action to direct analyte flow and produce visible colour changes. Synthesized from biodegradable materials like cellulose, they are ideal for nontechnical users [16]. Silver nanoparticles (AgNPs) have emerged as a superior alternative for colorimetric sensing due to their high extinction coefficients, enhancing optical brightness and sensitivity in UV-visible detection [17]. Biochemical assays such as the lead chromate test and the sodium hydroxide test are also employed to detect heavy metals, specifically lead and mercury, in milk samples. Thus, in this study, we proposed a paper-based colorimetric sensor based on green synthesized silver nanoparticles (Ag NPs) from leaf extract of *Hemigraphis colorata* for the detection of heavy metal ions in milk.

EXPERIMENTAL

Preparation of plant extract: Fresh leaves of *H. colorata* were collected, washed thoroughly in tap water and shade-dried for a few days. It was then finely powdered and stored for extract preparation. The plant extract was prepared by dissolving 5 g of powder into 100 mL of distilled water. It was then heated at 50 -60 ºC for 20-25 min in a hot plate magnetic stirrer. The filtrate was then filtered using muslin cloth twice to remove impurities and stored in cold conditions for further use.

Biosynthesis of AgNPs: A 10 mM silver nitrate solution was freshly prepared and stored in a dark condition. For the biosynthesis of silver nanoparticles, plant extract and silver nitrate solution were combined in a ratio of 1:10 and the pH of the mixture was adjusted to alkaline by gradual titration with NaOH. A colour change from light brown to wine red occurred within minutes, indicating nanoparticle formation. The supernatant was then extracted from the solution after it had been centrifuged for 15 min at 10,000 rpm. The resulting pellet was resuspended in distilled water and centrifuged again at 10,000 rpm for 15 min, with this washing step repeated twice. Afterwards, the pellet was cleaned with 100% ethanol and allowed to dry for 1 h at 50 ºC in a hot air oven. Finally, the dried nanoparticle sample was finely ground using a micropestle and stored in an Eppendorf tube for further characterization.

Collection of milk samples: Four different milk samples were collected from the local markets of Bengaluru city, India. One natural cow milk, two commercial milk samples and one milk powder were collected and named M1, M2, M3 and M4 respectively and stored in the cold room for further use.

Characterization: The X-Ray Diffraction (XRD) patterns were recorded on PANalytical X-ray diffractometer using Cu$K\alpha$ radiation (λ = 1.5406 Å) operated at 50 kV and 100 mA. The experiments performed in the diffraction angle range of 2θ = 20-80º. The UV-Vis measurements were carried out using Shimadzu UV1700 pharmaspec UV-visible spectrophotometer. Infrared analysis were performed by a Nicolet Nexus 670 Fourier transform infrared spectrometer in a wavenumber range from 4000 to 400 cm^{-1}. The surface morphological characterization was performed by SEM (Jeol-JSM 6701–F), whereas the EDX analysis was performed on a Hitachi SU6600 FE–SEM equipped with an EDX attachment.

Sample preparation of AAS analysis: A 50 mL of 24% TCA was added to 5 mL of each milk sample in the volumetric flask to help in the milk sample's digestion. The milk samples were shaken at 5 min intervals for 30 min and then each sample was centrifuged at 4000 rpm for 5 min. It was then filtered using filter paper and stored in a stopper container. These acid-digested milk samples were used for the analysis of AAS with the help of Perkin-Elmer Analyst 300 AAS (Perkin-Elmer SK) instrument.

Biochemical tests for the detection of heavy metals

Potassium chromate test for lead ions (Pb^{2+}): For each milk sample, a confirmatory test of lead was performed. The confirmatory test of lead was done by adding a few drops of dil. HCl to the sample and to the precipitate formed, potassium chromate solution was added. A yellow precipitate of lead(II) chromate ($PbCrO_4$) forms, confirming lead in the sample. The colour change was observed with the addition of AgNPs. The same was repeated with lead-spiked milk samples. A 0.1 M lead acetate solution was taken as control and its colour change was observed with the addition of AgNPs solution.

Sodium hydroxide test for mercuric ions (Hg^{2+}): The confirmatory test for mercury was also performed for each milk sample. To a sample, few drops of NaOH was added. The formation of yellow HgO precipitate confirms mercury in the sample. The colour change was observed with the addition of nanoparticles. The same was repeated for milk samples spiked with mercury. The confirmatory test was done with the addition AgNPs and without the addition AgNPs to observe the change in the colour of the precipitate. A 0.1 M mercuric chloride acts as the control.

FTIR analysis of the precipitate formed in the biochemical test: The precipitate formed in the above-mentioned biochemical test was prepared for FTIR analysis to determine the functional group in the complex formed. Variations in colour changes

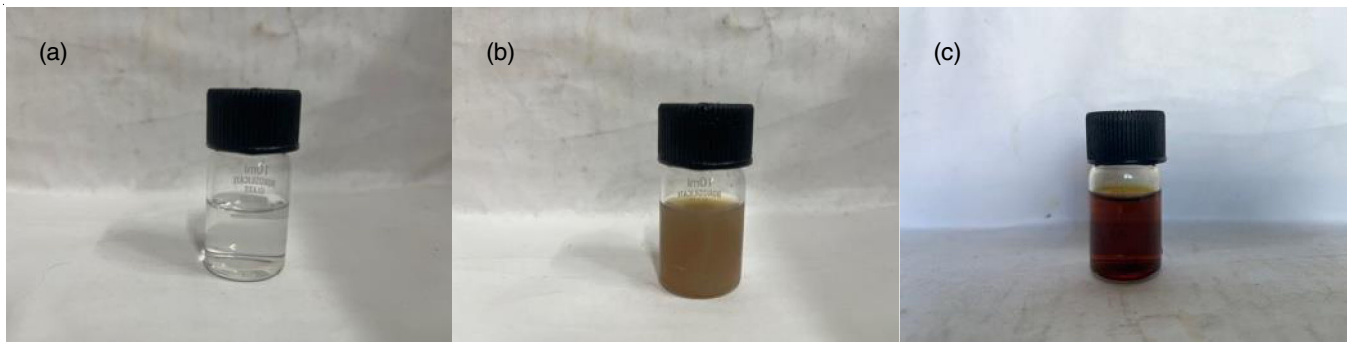


Fig. 1. (a) Silver nitrate solution, (b) plant extract and silver nitrate solution in 1:10 ratio respectively, (c) AgNPs solution

and precipitate formations were observed in each milk sample, interacted with AgNPs and other chemicals and the precipitate formed was analyzed.

Biochemical test for detection of heavy metals in milk using L-cysteine: Each of the milk samples was spiked with few drops of 0.1M lead acetate and mercuric chloride taken separately in two sets. To each of the samples, AgNP solution was added and mixed well. A few drops of 0.1M L-cysteine was added to each sample and the colour change was observed before and after the addition of L-cysteine.

Development of paper-based colorimetric sensor for the detection of lead and mercury: Whatman filter paper was cut into small strips of uniform size. It was coated with AgNP solution either by dip coating or spray coating method. It was then allowed to dry in room temperature or at low temperature in a hot air oven. The dried strips were then coated with L-cysteine to enhance their affinity towards mercury and lead ions. The coating can be done by dip coating or spray coating method and allowed to dry. These strips were then used for detecting heavy metals.

RESULTS AND DISCUSSION

A red wine-coloured AgNPs solution was obtained using leaf extract of *H. colorata,* as shown in Fig. 1. The pH of the AgNPs solution is alkaline and a stable red-wine colour was obtained after combining plant extract and AgNPs solution in 1:10 ratio. Thus, indicating the successful reduction of Ag^+ ions and formation of AgNPs solution.

UV-Vis spectral studies: The absorbance range was found to be 425 nm in UV-vis spectroscopy for the AgNPs solution is shown in Fig. 2. The deep red color arises from surface plasmon resonance (SPR), attributed to the presence of organic molecules capped with AgNPs. The stability of the AgNPs solution was confirmed by taking absorbance readings for four continuous months and the absorbance was found within the range of 400-500 nm.

FTIR spectral studies: The IR spectra of AgNPs solution were analyzed and two distinct peaks (Fig. 3) confirmed the presence of O-H stretch at 3300.28 cm^{-1} and C=O (carbonyl groups) & C=C stretch at 1636.57 cm^{-1}, which are due to to the presence of alcoholic/phenolic and aromatic compounds, respectively present in the leaf extract of *H. colorata*.

Morphological studies: The SEM image of green synthesized AgNPs at a magnification of 150000x and 50000x respectively are shown in Fig. 4. It has a size ranging from 30-35 nm and has spherical in shape. They appeared to be aggregated and polydisperse and a rough surface texture which influences its properties.

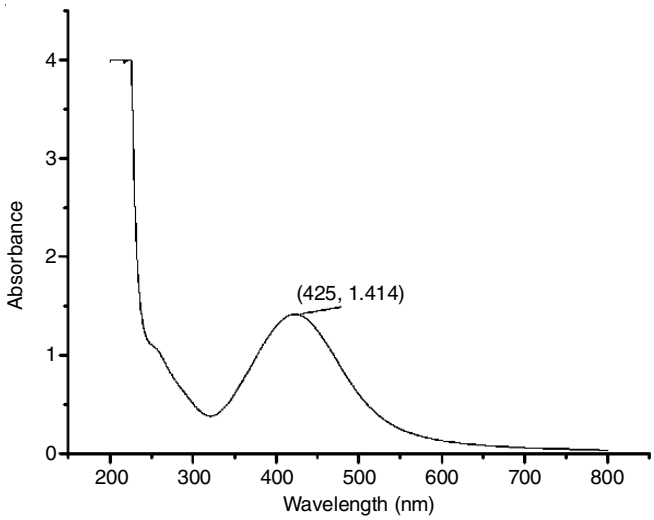


Fig. 2. UV-Vis spectrum of *H. colorata* mediated silver nanoparticles

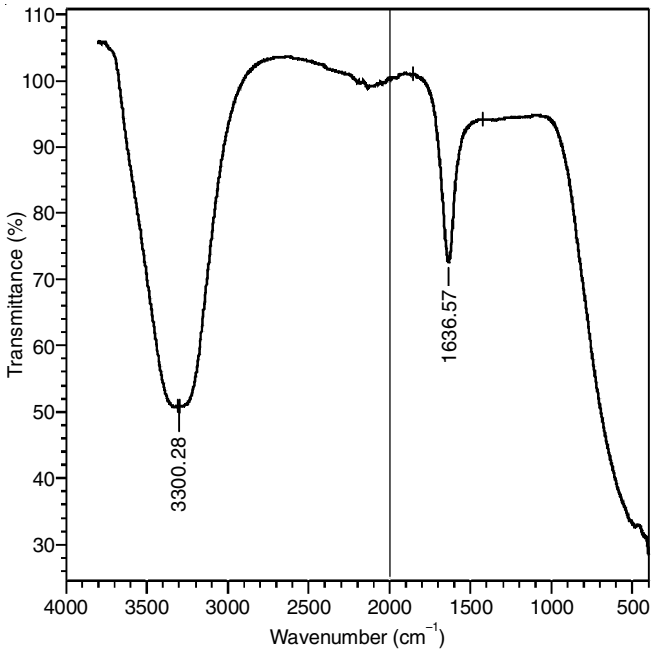


Fig. 3. FTIR spectrum of *H. colorata* mediated silver nanoparticles

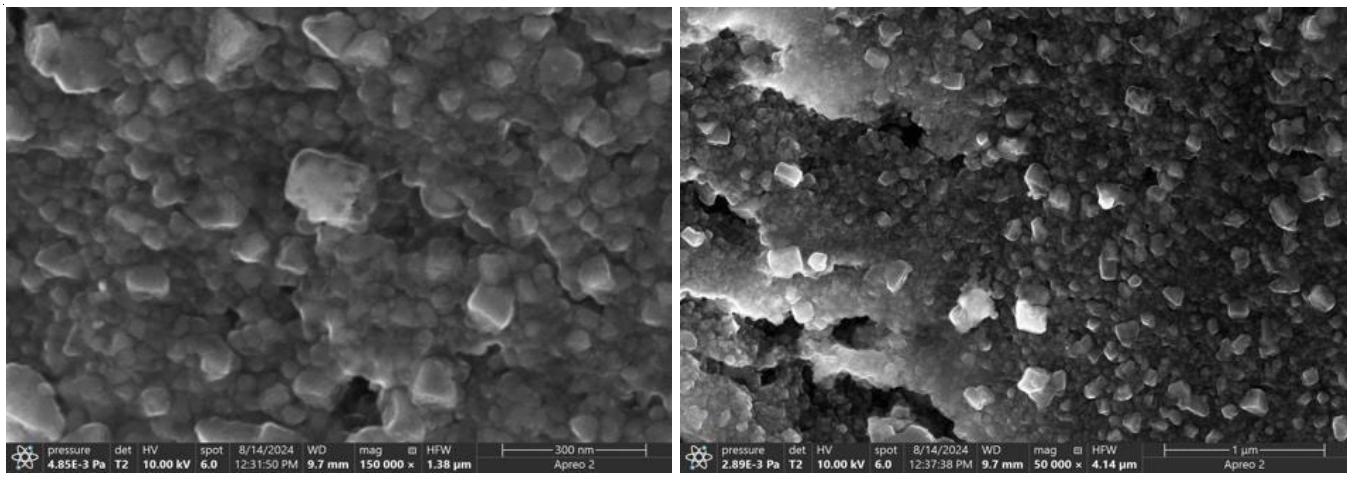


Fig. 4. SEM images of *H. colorata* mediated silver nanoparticles

SEM was paired with EDX and the elemental composition of silver nanoparticles was confirmed. AgNPs showed a strong signal at 3 keV as shown in Fig. 5, confirming the presence of silver majorly in the sample. The formation of other peaks are the oxide, sodium and carbon contamination that might have occurred during plant extract synthesis or it could be the residual elements formed during the synthesis.

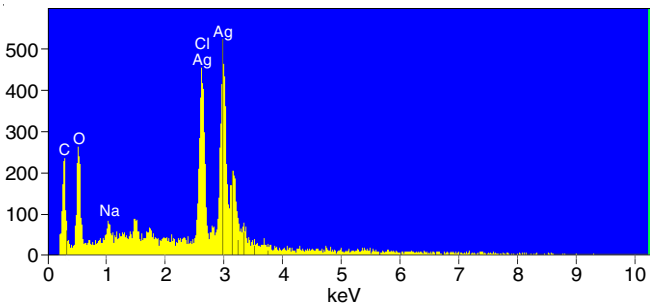


Fig. 5. EDX spectrum of *H. colorata* mediated silver nanoparticles

XRD studies: The AgNPs were characterized using XRD to determine their crystalline structure. The XRD pattern has four distinct peaks at 28.04º, 32.44º, 38.36º and 46.44º corresponding to the face-centred cubic (FCC) lattice of silver as shown in Fig. 6. The peaks at 38.36º and 46.44º indicates the AgNPs and peaks at 28.04º and 32.44º may be due to contamination or formation of other compounds.

Atomic absorption spectrometry (AAS): In AAS, none of the milk samples showed mercury content, however lead content was present in M1 and M2 sample with 0.4734 ppm and 0.0725 ppm concentrations, which are the natural and commercial samples respectively.

Biochemical tests for the detection of heavy metals: The confirmatory test for lead was conducted, revealing that the milk, when treated with dilute HCl and potassium chromate, had no typical precipitate. Upon the addition of AgNPs solution, the solution's colour changed to brownish-yellow. With the spiked milk samples, a yellow precipitate was formed and it turned light brown when the AgNPs solution was added, which is due to the plasmonic resonance shifts. The AgNPs may aggregate due to the Pb^{2+} ions, thus changing their colour (Fig. 7a).

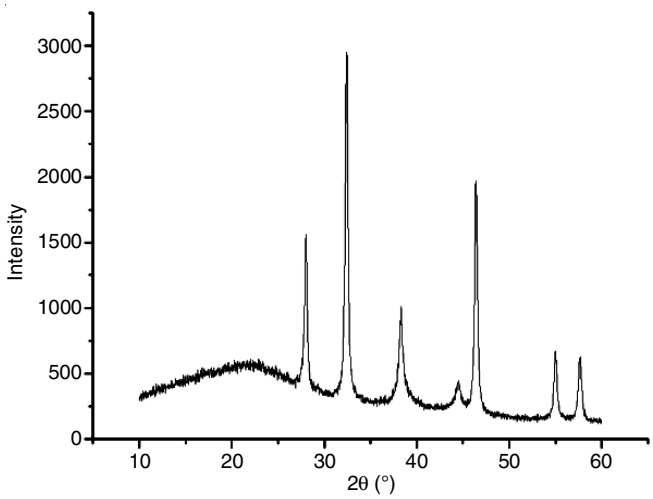


Fig. 6. XRD spectrum of *H. colorata* mediated silver nanoparticles

The confirmatory test for mercury detection in milk samples yielded no typical colour shift, indicating the absence of mercury (Fig. 7b), as confirmed by AAS analysis. A brownish-red colour was achieved upon the addition of AgNPs solution to the milk samples. All milk samples were infused with Hg^{2+} ions, resulting in the formation of a white precipitate upon the addition of NaOH. The addition of AgNPs solution resulted in a white turbidity solution and all the milk samples exhibited same distinctive alterations.

All the milk samples were spiked with Pb^{2+} ions. The compounds formed when milk containing lead ions interacts with HCl, potassium chromate and AgNP were analyzed with FTIR (Fig. 8). The FTIR peak at 1650-1630 cm^{-1} was observed in M2, M3 and M4 samples, confirmed the presence of C=O stretching, which might be formed due to the protein denaturation of milk samples. The peak at 1540 cm^{-1} is due to presence of the C-N amide II band, which might be due to the formation of lead-protein complexes. The peak at 1080-1000 cm^{-1} confirms the C-O stretching in the carbohydrates, which might be due to the interaction of phosphate groups and sugars in milk with lead. Another board peak at 840-830 cm^{-1} in M1 and M2 samples confirms the C-H bending, which might be due to the formation of metal coordination complexes involving aromatic comp-

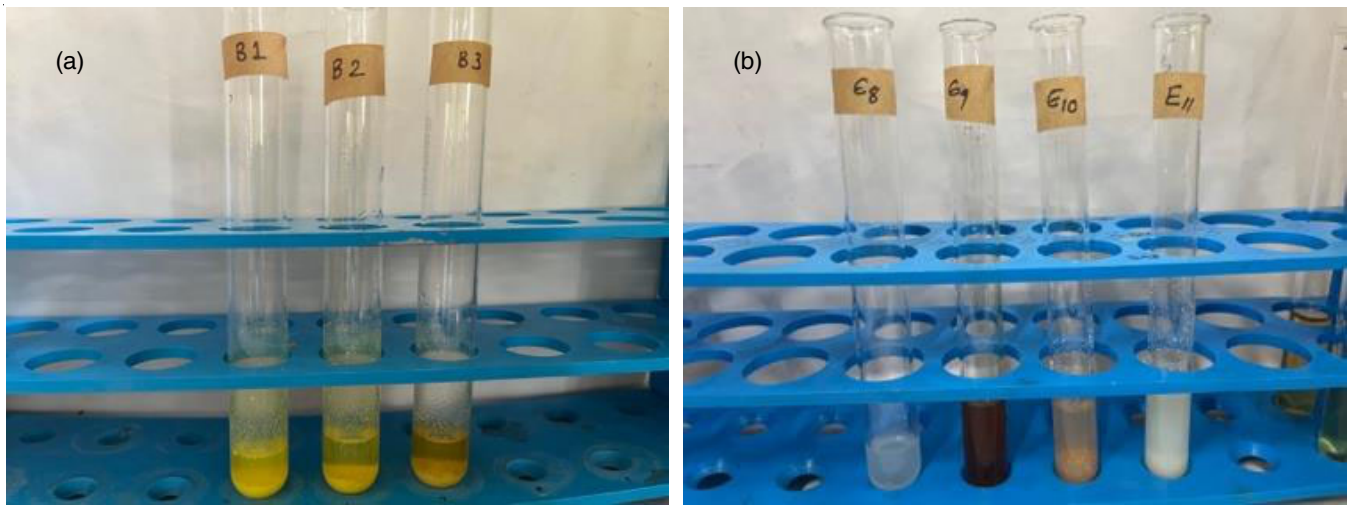


Fig. 7. (a) The confirmatory test of lead where B1 is M1 spiked with lead, containing dil. HCl and potassium chromate, B2 is M1 spiked with lead, containing dil. HCl, potassium chromate and AgNPs solution and B3 is M1 with HCl, potassium chromate and AgNPs solution; (b) the confirmatory test of mercury where E8 is M1 containing NaOH, E9 is M1 containing NaOH and AgNPs solution, E10 is M1 spiked with mercury, containing NaOH and E11 is M1 spiked with mercury, containing NaOH and AgNPs solution

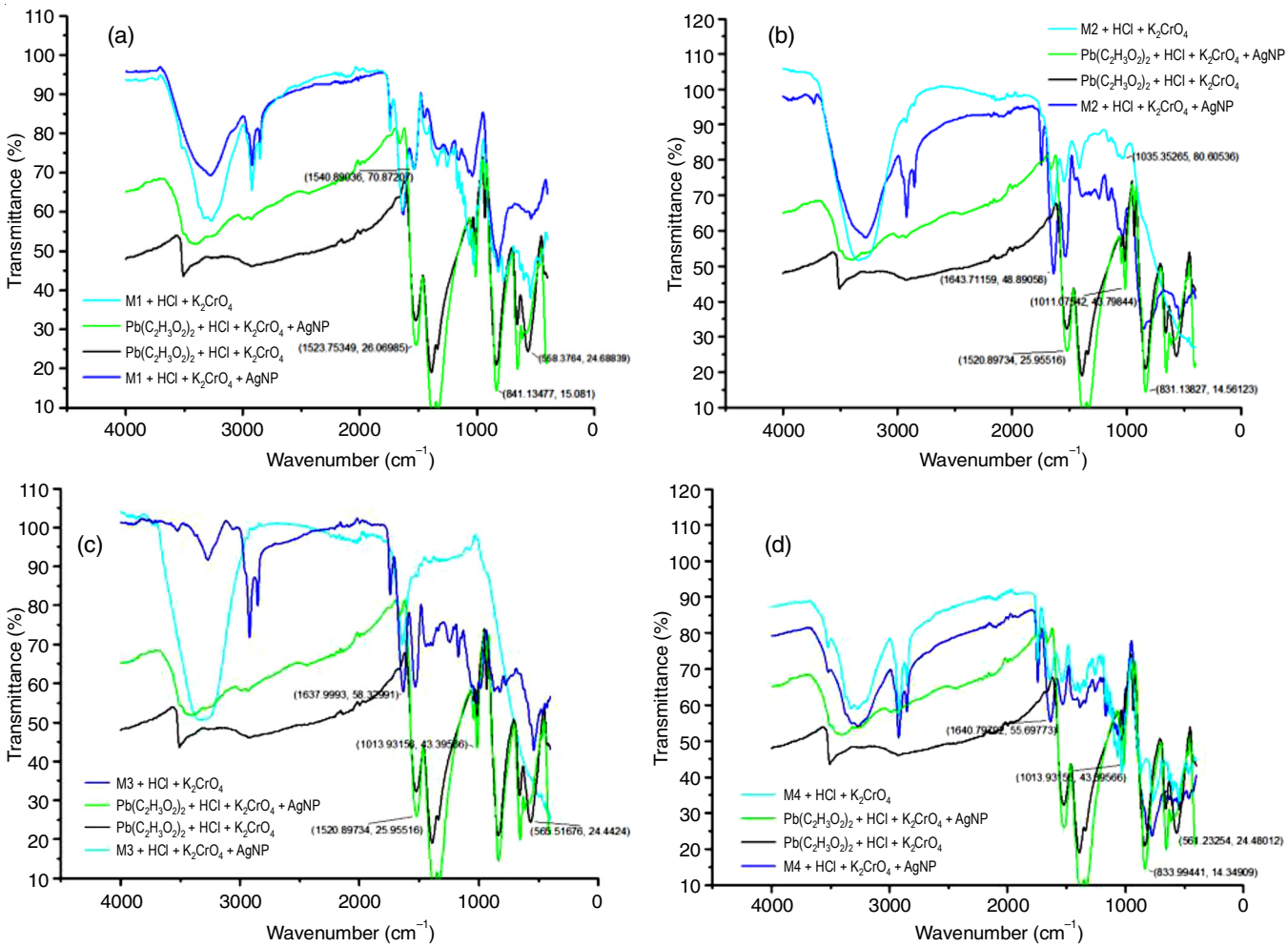


Fig. 8. FTIR analysis of biochemical test of milk spiked with lead; (a) M1, (b) M2, (c) M3, (d) M4

ounds. The peak at 570-550 cm^{-1} also confirmed the presence of metal coordination complexes like $PbCrO_4$.

To detect mercury in milk samples, all the samples were spiked with Hg^{2+} ions and the FTIR data of these samples reacting with NaOH and AgNPs solution are shown in Fig. 9. The peak at 3350-3280 cm^{-1} is common in all the milk samples, confirming the O-H stretching, which is due to the N-H stretching of amines. This hydroxyl group might be from the NaOH

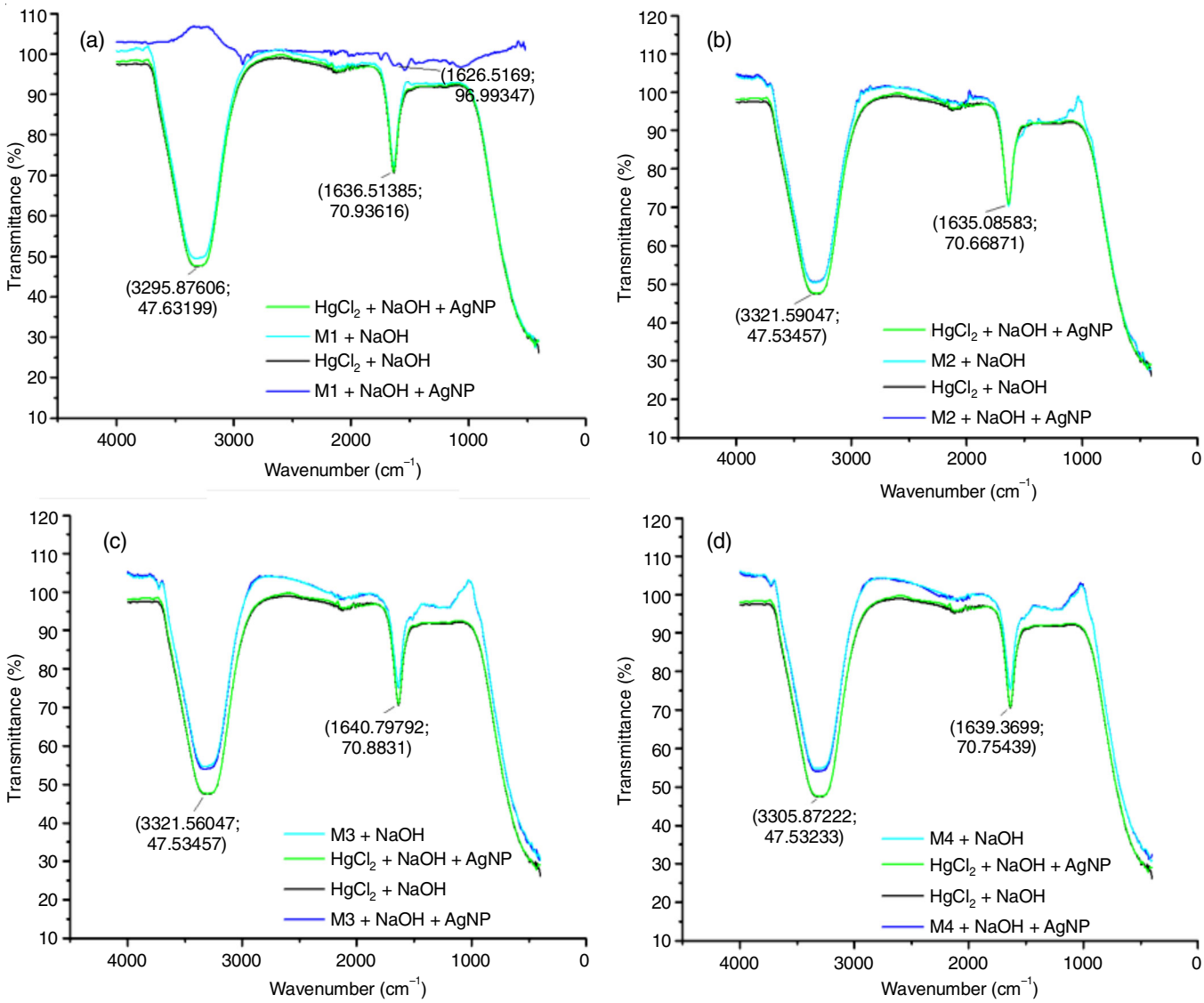


Fig. 9. FTIR analysis of biochemical test of milk spiked with mercury; (a) M1, (b) M2, (c) M3, (d) M4

reacting with milk proteins and N-H from the interaction of milk with mercury and AgNPs. The peak at 1640-1630 cm^{-1} in the data confirms the presence of C=O stretching of carbonyl groups or amides. This peak might be due to the interaction of mercury with proteins forming metal-protein complexes. The possible compounds formed could be Ag-protein complexes, mercuric hydroxide or mercury-protein complexes.

Biochemical test for detection of heavy metals in milk using L-cysteine: In the test tubes containing milk samples M1, M2, M3 and M4 spiked with lead, respectively, a brown colour appeared with the addition of AgNPs and with the addition of L-cysteine, a precipitate was formed. This precipitate may be formed due to the reaction of lead ions and L-cysteine. L-cysteine has a thiol group (-SH), an amino group ($-NH_2$) and

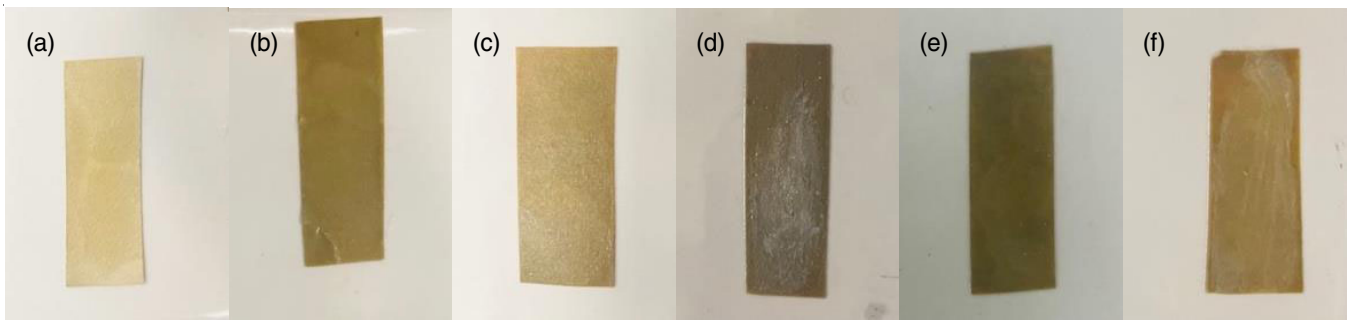


Fig. 10. Paper-based colorimetric sensor for detection of lead before incubation dipped in (a) distilled water, (b) 0.1 M lead acetate, (c) M1, (d) M2, (c) M3, (d) M4

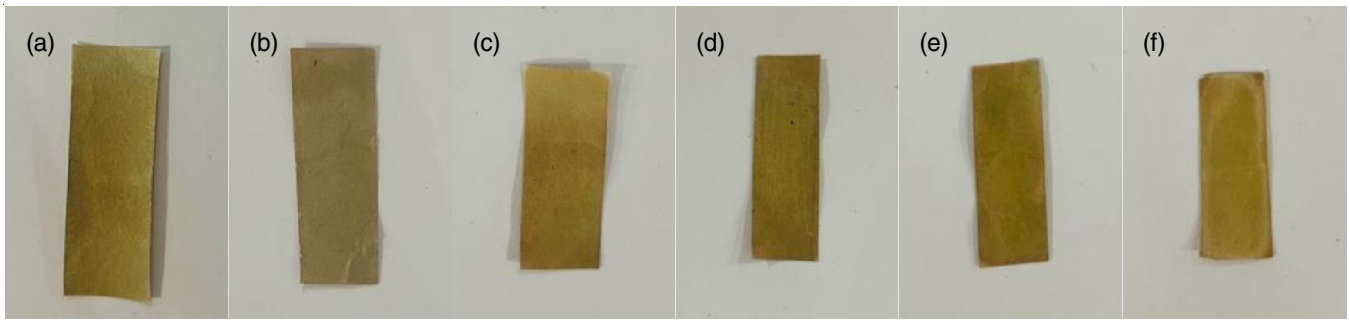


Fig. 11. Paper-based colorimetric sensor for detection of lead after incubation dipped in (a) distilled water, (b) 0.1 M lead acetate, (c) M1, (d) M2, (c) M3, (d) M4

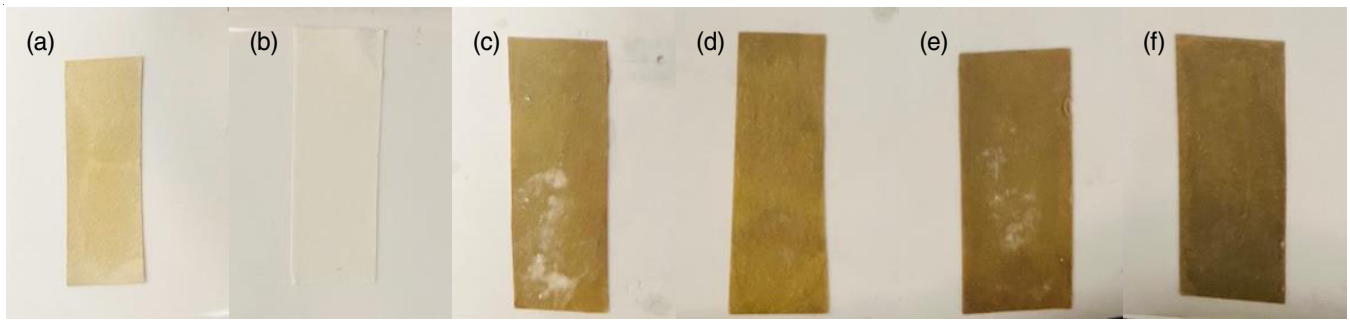


Fig. 12. Paper-based colorimetric sensor for detection of mercury before incubation dipped in (a) distilled water, (b) 0.1 M mercuric chloride, (c) M1, (d) M2, (c) M3, (d) M4

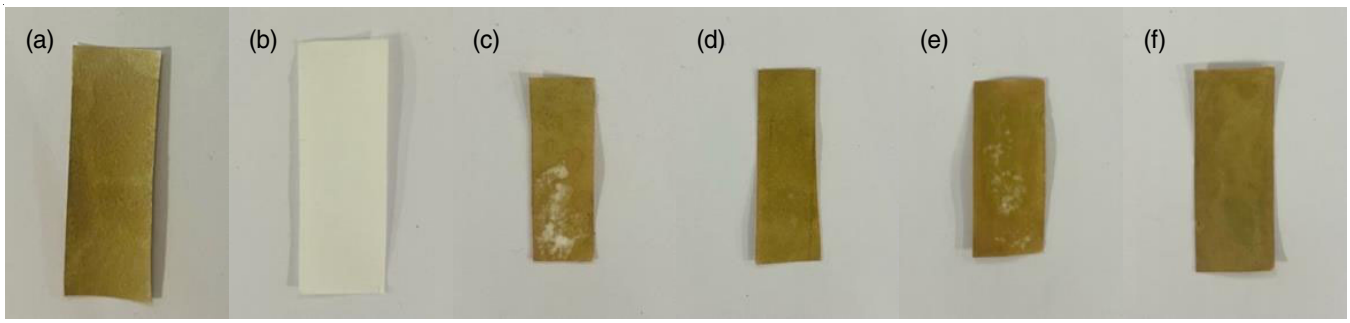


Fig. 13. Paper-based colorimetric sensor for detection of mercury after incubation dipped in (a) distilled water, (b) 0.1 M mercuric chloride, (c) M1, (d) M2, (c) M3, (d) M4

a carboxylic acid group (-COOH). These functional groups in L-cysteine can form bonds with the Pb^{2+} ions, thus forming a lead-cysteine complex which is insoluble and precipitates out of the solution. The precipitation process was enhanced by the AgNPs, which may interact with thiol groups due to their surface reactivity, resulting in the increased aggregation of lead ions with L-cysteine. This could enhance the efficacy of lead removal from milk. Another possibilities include the degradation of L-cysteine to produce sulfide ions, which may react with Pb^{2+} to form lead sulfide, an intractable black precipitate.

Several factors including the concentration of lead, the L-cysteine to AgNPs ratio and the milk's components affect the nature of precipitate. The milk samples spiked with mercury also a precipitate was formed after the addition of L-cysteine. This could be the result of the reaction of the thiol group in L-cysteine with Hg^{2+} ions forming an insoluble precipitate. There could be other possibilities like due to changes in the surface charge or binding interactions, AgNPs may form aggregate when Hg^{2+} is present. Larger AgNPs aggregates containing Hg^{2+}, L-cysteine and AgNPs may be formed with the help of L-cysteine acting as a mediator or it could be the formation of mercury sulfide.

Development of paper-based lead and mercury colorimetric test sensor: L-Cysteine coated paper was dipped in each milk sample spiked separately with lead and mercury ions and colour change was observed. There was no colour change in the strip dipped in distilled water. A red colour appeared on the strip dipped in 0.1 M lead acetate solution and a white patch appeared on the strip dipped in 0.1 M $HgCl_2$, which acts as the control. Lead ion-spiked milk turned red after a few hours, while mercury-spiked milk developed a white spot. This confirms the presence of heavy metals lead and mercury in milk samples and can be utilized as a reliable detection method. The results are shown in Figs. 10-13.

Conclusion

This study focuses on the detection of heavy metals in milk by biosynthesized AgNPs by using biochemical tests as well as paper-based colorimetric sensor based on green synthesized silver nanoparticles (AgNPs) from leaf extract of *H. colorata*. The development of paper-based colorimetric sensors coated with AgNPs solution and L-cysteine has paved the way for a reliable detection method for heavy metals in milk. The ability of AgNPs to interact with L-cysteine and heavy metals causes aggregation of molecules, which is responsible for the visible colour change. This has created a convenient method without relying on any advanced detection equipment. These methods act as an eco-sustainable tool for ensuring food safety around the globe and could be extended to various heavy metals and other animal products on further optimization.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

REFERENCES

1. B.L. Ilesanmi-Oyelere and M.C. Kruger, *Front. Nutr.*, **7**, 578702 (2020); https://doi.org/10.3389/fnut.2020.578702
2. A. Boudebbouz, S. Boudalia, A. Bousbia, S. Habila, M.I. Boussadia and Y. Gueroui, *Sci. Total Environ.*, **751**, 141830 (2021); https://doi.org/10.1016/j.scitotenv.2020.141830
3. C. Lopez, C. Cauty and F. Guyomarc'h, *Dairy Sci. Technol*., **95**, 863 (2015); https://doi.org/10.1007/s13594-015-0263-0
4. M. Singh, S. Ranvir, R. Sharma, K. Gandhi and B. Mann, *Indian J. Dairy Sci.*, **72**, 608 (2020); https://doi.org/10.33785/IJDS.2019.v72i06.005
5. A. Ismail, M. Riaz, S. Akhtar, J.E. Goodwill and J. Sun, *Toxin Rev.*, **38**, 1 (2019); https://doi.org/10.1080/15569543.2017.1399276
6. S. Anjum, S. Ishaque, H. Fatima, W. Farooq, C. Hano, B.H. Abbasi and I. Anjum, *Pharmaceuticals*, **14**, 707 (2021); https://doi.org/10.3390/ph14080707
7. N. Savage and M.S. Diallo, *J. Nanopart. Res.*, **7**, 331 (2005); https://doi.org/10.1007/s11051-005-7523-5
8. N. Cao, R. Jin and C.A. Mirkin, *J. Am. Chem. Soc.*, **123**, 7961 (2001); https://doi.org/10.1021/ja011342n
9. M. Han, X. Gao, J.Z. Su and S. Nie, *Nat. Biotechnol.*, **19**, 631 (2001); https://doi.org/10.1038/90228
10. P.V. Kamat, *J. Phys. Chem. B*, **106**, 7729 (2002); https://doi.org/10.1021/jp0209289
11. B.N. Salman, M.M. Gheidari, A.Y. Nejad, H. Zeighami, A. Mohammadi and S.B. Shabestari, *Med. J. Islam. Repub. Iran*, **36**, 154 (2022); https://doi.org/10.47176/mjiri.36.154
12. M.G. Heinemann, C.H. Rosa, G.R. Rosa and D. Dias, *Trends Environ. Anal. Chem.*, **30**, e00129 (2021); https://doi.org/10.1016/j.teac.2021.e00129
13. M. Elangovan, D. Ramachandran and K. Rajesh, *Lett. Appl. NanoBioSci.*, **10**, 2646 (2021); https://doi.org/10.33263/LIANBS104.26462654
14. S. Nayak, L.C. Goveas, P.S. Kumar, R. Selvaraj and R. Vinayagam, *Food Chem. Toxicol.*, **167**, 113271 (2022); https://doi.org/10.1016/j.fct.2022.113271
15. G. Alberti, C. Zanoni, L.R. Magnaghi and R. Biesuz, *Chemosensors*, **9**, 305 (2021); https://doi.org/10.3390/chemosensors9110305
16. N. Ratnarathorn, O. Chailapakul, C.S. Henry and W. Dungchai, *Talanta*, **99**, 552 (2012); https://doi.org/10.1016/j.talanta.2012.06.033
17. M. Taufiq, W.T. Eden, W. Sumarni and M. Alauhdin, *J. Phys. Conf. Ser.*, **1918**, 032002 (2021); https://doi.org/10.1088/1742-6596/1918/3/032002