Environmentally Benign Synthesis of Silver Nanoparticles for the Colorimetric Detection of Melamine in Milk Samples

A thesis submitted towards the partial fulfilment of the requirements for the degree of Master of Technology in Food Technology and Biochemical Engineering course affiliated to Faculty of Engineering and Technology, Jadavpur University

Submitted by

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Certificate of Recommendation

I hereby recommend the thesis entitled ***"Environmentally Benign Synthesis of Silver Nanoparticles for the Colorimetric Detection of Melamine in Milk"***, is carried out under my supervision by **Arnab Saha** of Registration No. **160271** of **2021-2022**. The thesis has been evaluated by me and found satisfactory. It is therefore, being accepted in partial fulfilment of the requirement for awarding the degree of Master of Technology in Food Technology and Biochemical Engineering course affiliated to Faculty of Engineering and Technology, Jadavpur University.

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Declaration of Originality and Compliance of Academic Ethics

I hereby declare that this thesis contains literature survey and original research work by the undersigned candidate, as part of my Master of Technology in Food Technology and Biochemical Engineering. All information in this document have been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referred all material and results that are not original to this work.

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The foregoing thesis is hereby approved as a creditable study in **Master of Technology in Food Technology and Biochemical Engineering** and presented in a manner satisfactory to warrant its acceptance as a prerequisite to the degree for which it has been submitted. It is understood that by this approval the undersigned do not necessarily endorse or approve any statement made, opinion expressed or conclusion drawn therein but approve the thesis only for the purpose for which it is submitted.

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Abstract

The rapid and sensitive detection of melamine, a nitrogen-rich chemical compound commonly used in food adulteration, remains a significant challenge in food safety and quality control. In this study, we present a novel colorimetric method for the detection of melamine using silver nanoparticles (AgNPs) synthesized via eco-friendly approach. The unique optical properties of AgNPs, specifically their Localised Surface Plasmon Resonance (LSPR), were exploited to develop a simple and cost-effective detection technique. The synthesis of AgNPs was achieved using a plant extract as a reducing and stabilizing agent, ensuring environmentally friendly and biocompatible nanoparticle production. The resulting AgNPs exhibited distinct LSPR characteristics in the visible spectrum, with a prominent absorption peak at around 416 nm. Upon exposure to melamine, the interaction between melamine molecules and AgNPs induced a shift in the LSPR wavelength, resulting in a visually observable colour change from light yellow to straw yellow colour.

The colorimetric detection method exhibited high sensitivity, with a detection limit in the low Nano-molar range. Additionally, the assay showed excellent selectivity for melamine in the presence of potential interferents, demonstrating its applicability in complex matrices. The response time of the assay was rapid, allowing for real-time, on-site detection of melamine without the need for specialized equipment. Its simplicity, sensitivity, and eco-friendly nature make it an attractive option for addressing food safety concerns, ensuring consumer protection, and safeguarding the integrity of the food supply chain. Further development and optimization of this technique could pave the way for its widespread adoption in various industries concerned with melamine contamination.

Keywords: Melamine, Green synthesized silver nanoparticles, Spectrophotometric analysis, Milk, Detection

List of Abbreviations

HPLC: High-performance Liquid Chromatography

LC-MS: Liquid Chromatography /Mass Spectrometry

GC-MS: Gas Chromatography/Mass Spectrometry

ELISA: Enzyme-Linked Immunoassay

CE: Capillary Electrophoresis

SPR: Surface Plasmon Resonance

CCD: Charged Couple Device

LSPR: Localized Surface Plasmon Resonance

SERS: Surface Enhanced Raman spectroscopic

nm: Nanometre

RIU: Refractive Index Unit

WHO: World Health Organization

FAO: Agriculture Organization

CAC: Codex Alimentarius Committee

FDA: Food and Drug Administration (US),

EC: European Community

MRL: Maximum Residue Limits

FSSAI: Food Safety and Standards Authority of India

PDA: Polydiacetylene

NPs: Nanoparticles

AgNPs: Silver Nanoparticles

AuNPs: Gold Nanoparticles

LOD: Limits of Detection

mg/L: Milligram per Litre

ppm: Parts per Million

UV–Vis spectra: Ultra Violet Visible Spectrophotometer

Ml: Millilitre

mM: Mill mole per litre

g/ml: Gram per Millilitre

RSD: Relative Standard Deviation

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Chapter 1:
Introduction

Introduction

Facts about Food Adulterants:

Food adulteration is a pervasive issue worldwide, with significant implications for public health, consumer trust, and the integrity of the food supply chain. Adulterants are substances intentionally added to food products with the aim of increasing profitability, enhancing appearance, or extending shelf life. This illicit practice has been documented throughout history and continues to pose serious challenges to food safety and quality control. [1]

A wide range of substances can be used as food adulterants, including but not limited to: [2][3][4]

Melamine: Melamine, a nitrogen-rich compound, is known for its notorious role in food adulteration. It has been illicitly added to dairy products to artificially inflate protein content, leading to significant health concerns.

Food Dyes: Synthetic food dyes are often used to enhance the colour of food products, especially in candies, beverages, and processed foods. Some of these dyes have raised health concerns, particularly in relation to their potential impact on hyperactivity in children.

Artificial Sweeteners: While approved for use in many foods, artificial sweeteners have been subject to adulteration in some instances, posing risks to consumers who expect natural or regulated sugar substitutes.

Adulteration of Edible Oils: Substances such as non-edible mineral oils or cheaper vegetable oils have been used to adulterate edible oils, compromising the nutritional quality of these products.

Counterfeit Alcohol: The adulteration of alcoholic beverages with toxic or cheaper substitutes, such as methanol, poses severe health risks and is a common problem in the production of illicit spirits.

Dilution of Fruit Juices: Some fruit juices are diluted with water or cheaper juices to increase volume without consumers' knowledge.

Caffeine in Herbal Supplements: Certain herbal supplements have been found to contain undisclosed caffeine, potentially leading to health issues for those seeking caffeine-free alternatives.

Food adulteration not only deceives consumers but can also have dire health consequences, as it may introduce harmful substances or mask the presence of contaminants. Regulators and food safety authorities globally continuously monitor and enforce standards to combat food adulteration and protect public health.

Reason for using Nanotechnology to Detect Food Contaminants:

Nanotechnology has emerged as a game-changer in the field of food safety due to its unparalleled ability to detect and mitigate food contaminants. The integration of nanoscale materials and devices offers numerous advantages, including enhanced sensitivity, rapid detection, and increased selectivity. The small size and high surface area of nanoparticles enable them to interact with contaminants at the molecular level, allowing for the detection of even trace amounts. Additionally, nanotechnology-based sensors can be designed to target specific contaminants or pathogens, reducing the risk of false positives and providing real-time monitoring. This revolutionary approach to food safety not only safeguards public health but also ensures the integrity of the global food supply chain. As a result, nanotechnology continues to drive innovation in food safety, offering more reliable and efficient methods for contaminant detection. [5]

Conventional methods such as high-performance liquid chromatography (HPLC), liquid chromatography /mass spectrometry (LC-MS), gas chromatography/mass spectrometry (GC-MS), ELISA, capillary electrophoresis (CE), and other biological immunological assays have been used for the detection of chemical and biological contaminants. The advantages of these methods are highly sensitivity with good accuracy, and their disadvantages are they are complicated and time-consuming and need expensive instruments with trained personnel for specific skills, which strongly limit their application.

Therefore, novel methods that are simple, rapid, selective, and sensitive are greatly required as an alternative. Nano sensors could be used as a more efficient and effective tool in the detection of chemical and biological contaminants. Various Nano sensors and Nano-biosensors used in the detection of chemical and biological contaminants and here in this paper we focus on the use of nanotechnology or nanoparticles to overcome the issues of conventional method to detect food contaminants. [6]

Concept of Surface Plasmon Resonance:

Surface plasmon resonance (SPR) technique is an optical method for measuring the refractive index of very thin layers of material adsorbed on a metal. A fraction of the light energy incident at a sharply defined angle can interact with the delocalized electrons in the metal film (plasmon) thus reducing the reflected light intensity. The SPR technique is based on the fact that, at certain conditions, surface plasmon on a metallic film can be excited by photons, thereby transforming a photon into a surface plasmon and it depend on the refractive index of the adsorbate. [7]

The most common geometrical setup (the Kretschmann configuration) of SPR can be seen in the below **Figure 1**. The incoming light is generally located on the opposite side of the metallic film than the adsorbate because photons cannot excite surface plasmon on the surface being hit.

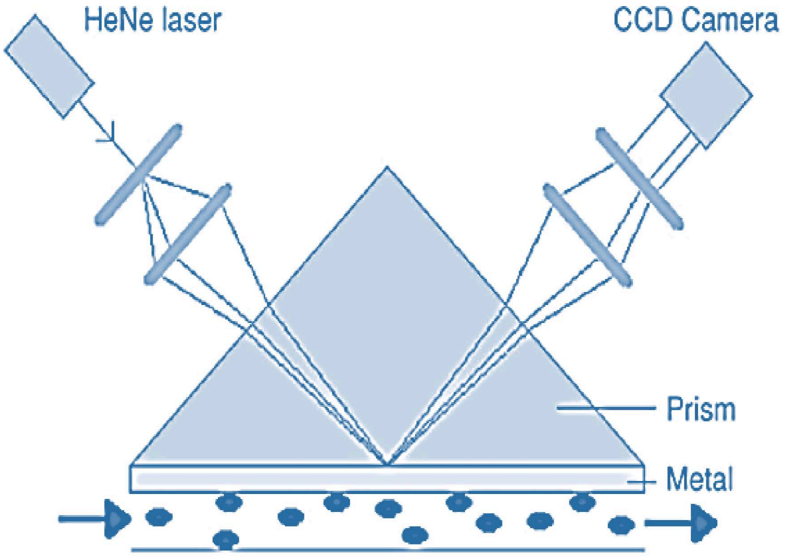


Figure 1: The most common geometrical setup (the Kretschmann configuration) of SPR. The incoming light is located on the opposite side of the metallic slab than the adsorbate.

The photons induce an evanescent light field into the metallic film. Normally no transport of photons takes place through this field, but photons incident at a certain angle are able to pass through the field and to excite surface plasmon on the adsorbate side of the metallic film. Whenever a plasmon is excited, one photon disappears producing a dip in reflected light at that specific angle. The angle, which is dependent on refractive index of the adsorbate, is measured with a charged couple device (CCD) chip. [8]

In the case of protein adsorption, the difference between the refractive index of the buffer (i.e., water) and the refractive index of the adsorbate can be easily converted into mass and thickness of the adsorbate as all proteins have almost identical refractive indices. The precise angle of incidence at which this occurs is determined by a number of factors, but in the Biacore system the principal determinant is the refractive index close to the backside of the metal film, to which target molecules are immobilized and addressed by ligands in a mobile phase running along a flow cell. If binding occurs to the immobilized target, the local refractive index changes, leading to a change in SPR angle, which is monitored in real time by detecting changes in the intensity of the reflected light, producing a senso-gram. The advantages of the Kretschmann configuration are that it is not necessary to shine light through the adsorbate and that it is easy to build. [9]

Concept of Localised Surface Plasmon Resonance:

The interaction of light with noble metal nanoparticles produces a collective oscillation of conduction band electrons known as the localized surface plasmon resonance (LSPR). Only materials with a negative real and small positive imaginary dielectric constant are capable of supporting surface plasmons. The most common materials used are gold and silver, although other metals such as copper and aluminium also exhibit plasmon resonance. When the incident electromagnetic field matches that of the oscillating electrons on the surface of the nanoparticle, a resonance condition is met. [10] It explained in below **Figure 2** with proper schematics.

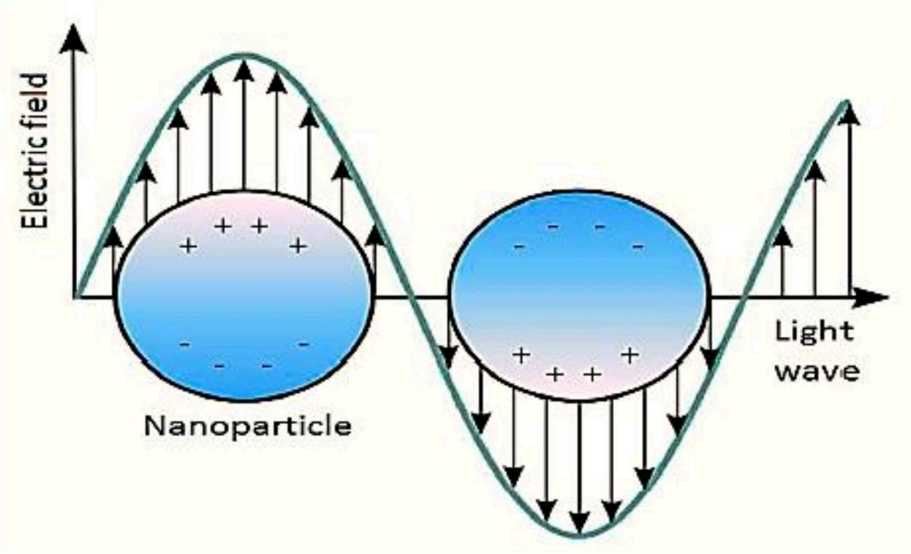


Figure 2: The localized surface plasmon on a nanoparticle surface

This resonant oscillation produces large, wavelength-selective increases in absorption, scattering, and electromagnetic field at the nanoparticle surface. The increases in absorption and scattering have been utilized towards LSPR biosensing. The increases in electromagnetic fields have also proven very useful in Surface Enhanced Raman spectroscopic (SERS) detection of biological analytes. However, this article is focusing on Food Contaminants detection using LSPR sensing. [11]

In order to understand the factors that affect the large increase in absorption and scattering when resonance occurs, Mie theory is often evoked [12]. Mie theory is an analytical solution to Maxwell's equations with spherical boundary conditions, which is used to describe the extinction spectra of a given nanoparticle. In order to more accurately calculate the dielectric constants at the different wavelength values and extend this theory to more complex shapes, the Modified Long Wavelength Approximation (MLWA) of Mie theory is used [13]:

$$C_{ext} = \frac{24\,\pi^2 R^3 \varepsilon_m^{\frac{3}{2}} N}{\lambda\, ln(10)} \frac{\varepsilon_i}{(\varepsilon_r + \chi\varepsilon_m)^2 + \varepsilon_i^2} \qquad \text{---(i)}$$

Where,

R= the radius of the particle,
λ = wavelength of the incident light,
ε_m =dielectric constant of the surrounding medium,

$\varepsilon = \varepsilon_r + i\varepsilon_i$ = complex dielectric constant of the bulk metal,
N = electron density,
χ = accounts for the shape of the particle.

This "shape factor", χ, models the particles as an ellipse and is proportional to a/b where a and b are the minor and major axis respectively of the ellipse. As shown in Equation (1), many factors such as shape of the nanoparticle, wavelength of incident light, type of material, and the surrounding media influence the absorption and scattering processes.

In fact, the large effect of the surrounding dielectric constant on the extinction spectra of the plasmonic nanoparticle has been the basis of much of the work carried out in the field of LSPR biosensing. When a biological analyte binds to the surface of the nanoparticle, a change in refractive index at the nanoparticle surface is induced, which in turn shifts the LSPR peak frequency. This shift of LPSR frequency is also affected by the makeup of the nanoparticle and its shape. Silver, having the largest negative real dielectric constant of all the plasmonic materials, is the most sensitive to changes in local refractive index. In addition, nanoparticles of asymmetric shape are also more sensitive to changes in biomolecular surface binding than spherical colloids. The shift in LSPR frequency upon adsorbate binding has been described by the following relation: [14,15]

$$\Delta\lambda = m\,(\Delta n)\,[1 - exp(\frac{-2d}{l_d})] \quad \text{--(ii)}$$

Where,

m=refractive index sensitivity,
Δn =change in refractive index induced by the adsorbate,
d =effective adsorbate layer thickness,
l_d = electromagnetic field decay length (approximated as an exponential decay).

Thus, two key variables that determine the size of the LSPR shift observed is the difference in refractive index of the absorbate relative to solution (Δn) and the size of the analyte (d) that is binding to the nanoparticle surface. The refractive index sensitivity (m) is often obtained by taking the slope of a plot of LSPR frequency *versus* refractive index, which is predicted to be linear (within a relatively small range of index of refraction) by the Drude model of the electronic structure of metals [16].

Last, the electromagnetic field decay length, l_d, which can also affect the LSPR shift observed, has been shown to be sensitive to the shape of the nanoparticle. This electromagnetic field decay length for many nanoparticles (50–100 nm in diameter) is similar in size to that of a protein molecule, 5–10 nm, which allows for the unique sensitivity of noble metal nanoparticles to sense the binding of biological molecules to the surface [17,18].

Difference between SPR & LSPR:

Surface Plasmon Resonance [SPR]	Localised Surface Plasmon Resonance [LSPR]	Reference
SPR can be excited on the metallic films which have several approaches as the Kretschman and Otto prism coupler, optical waveguides coupler, diffraction gratings, and optical fibre coupler.	LSPR can be excited on metallic nano-particles, which both can induce a strong enhancement of electromagnetic filed in the near-field region (resonance amplification), leading to a extensive application in surface-enhanced Raman scattering (SERS), fluorescence enhancement, refractive index measurement, biomolecular interaction detection, and so on.	19, 20, 21
Traditional SPR uses a continuous gold film	LSPR is created through metal nanoparticles, usually silver and gold.	22, 23
Traditional SPR technique doesn't show much changes or shift in absorbance peak.	A powerful resonance absorbance peak is produced in the visible range of light by LSPR.	24, 25
In SPR technique is greater susceptible to bulk effects and less susceptible to molecular binding, which means greater interference from bulk signals.	With a more compact sensing volume, the LSPR technique is less susceptible to bulk effects and more susceptible to molecular binding, which means less interference from bulk signals.	26, 27
Sensitivity of traditional SPR instruments is 1 RU/pg/mm^2	While that of LSPR is 6 RU/pg/mm^2.	28

Effect of Size of Nanoparticles:

For metal nanoparticles the properties of LSPR depends strongly on the particle size. According to Mie theory, for R much lesser than the wavelength of light (2πR<<λ), the magnitude of the scattering cross-section is proportional to R, while absorption is proportional to R. Because of the pre-factors, for the smallest particles, LSPR extinction is dominated by absorption, and as particle size increases, scattering takes over.

For gold nanospheres, this transition occurs at around 80 nm in particle diameter [29]. This is in addition to the well-known dependence of plasmon resonance wavelength on particle size. For gold nanospheres, the LSPR wavelength can be tuned over 60 nm by varying particle size between 10 and 100 nm [29].

This effect was noted as early as 1909 by Gustav Mie's contemporary Richard Zsigmondy, who carried out early dark field microscopy studies of gold colloid has shown in **Picture 1**. The plasmon resonance line width also varies with particle size, due to a combination of inter-band transitions, which contribute to increased line width for small particles, and higher order (non-dipole) plasmon modes, which contribute to increased line width for larger particles [29].

Picture 1: Size dependence of gold colloid optical properties.
(a) 1909 illustration of dark field scattering of gold colloid of various sizes.
(b) Modern photograph of similar colloid solutions (Ted Pella Co.).

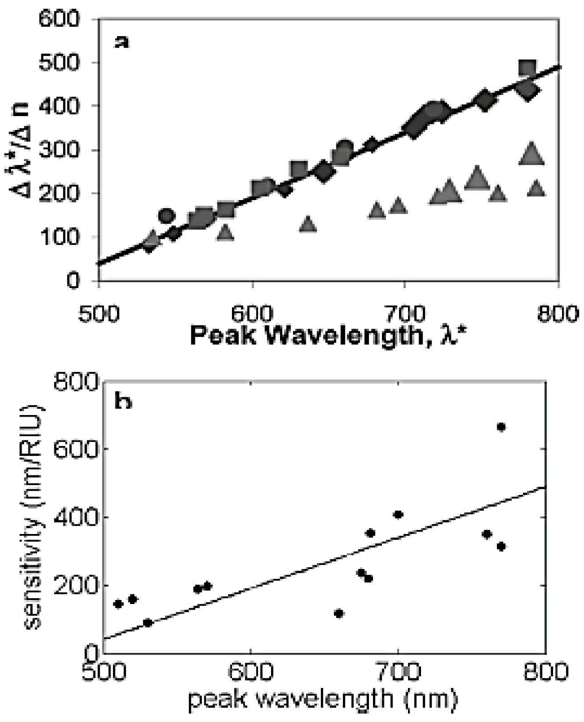
Effect of Shape of Nanoparticles:

The effect of particle shape on LSPR properties was demonstrated in the work of Mock et al., in which the spectra of silver nanoparticles of different shapes (spheres, triangles, and cubes) but similar volume were correlated with their structure [30, 31]. In addition, the particles' refractive index sensitivity was characterized at the single-particle level, and it was determined that silver Nano triangles had a much higher sensitivity (350 nm/RIU) than spheres (160 nm/RIU) [32].

As another example, Sun et al. demonstrated the difference in refractive index sensitivity between gold nanospheres and Nano-shells of the same diameter. They showed that the nano-shells had a much higher sensitivity (409 nm/RIU) as compared to the spheres (60 nm/RIU) [33]. Particles with sharp tips (Nano triangles, bipyramids) exhibit especially high refractive index sensitivities [34,35]. As seen in theoretical work from Miller and Lazarides, nanoparticle refractive index sensitivity varies linearly with plasmon resonant wavelength [30].

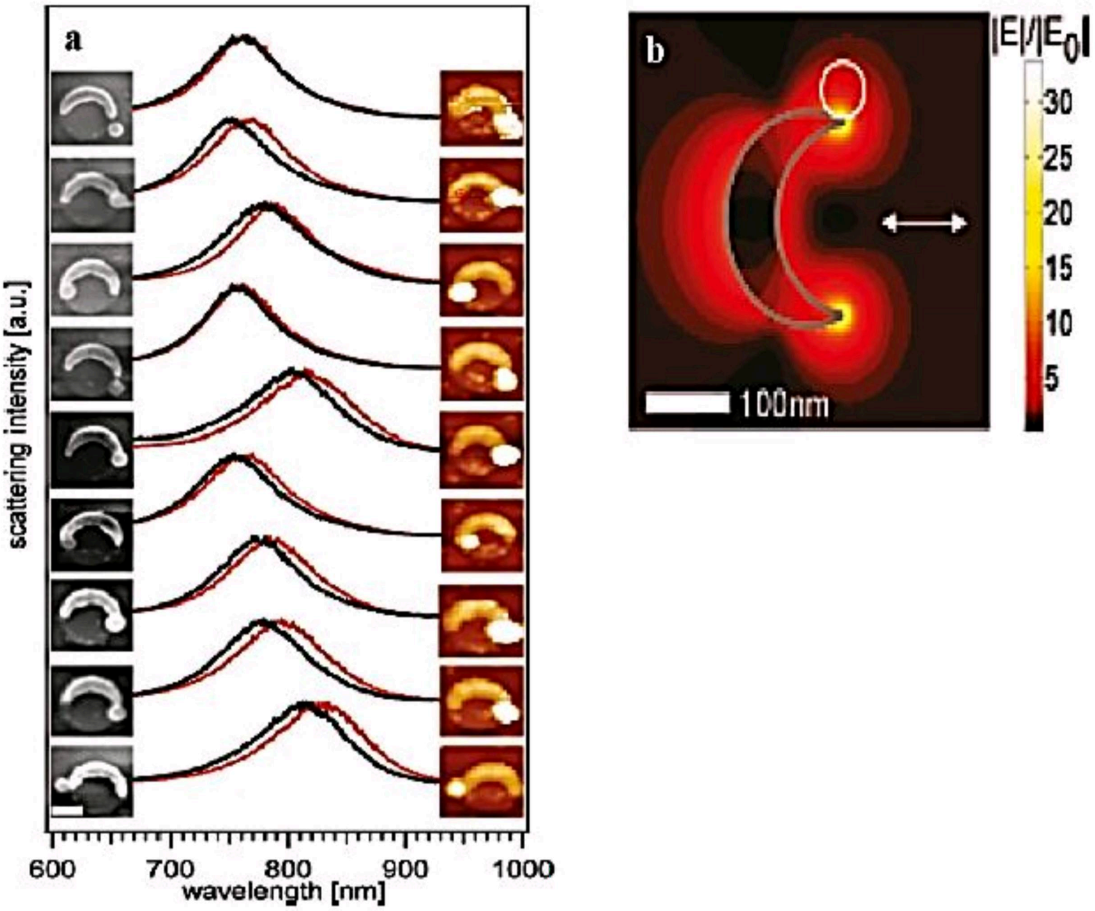
As seen in **Graph 1**, hollow nanoshells, nanodiscs, and nanocylinders all fall on the same line in a plot of sensitivity versus peak wavelength. This implies that intrinsic shape effects are much smaller than the plasmon resonance wavelength effect.

However, for nanoshells with a dielectric core, the slope decreases relative to that of solid metal nanoparticles, due to the plasmon damping effect of the dielectric material [30].



Graph 1:
(a) Theoretical predictions of refractive index sensitivity for hollow nanoshells, dielectric-core nanoshells, discs and cylinders of varying plasmon resonant wavelength.
(b) Sensitivity versus peak wavelength for the particles with solid line from overlaid.

In **Picture 2**, it is observed that, there are subtle differences between the bulk refractive index sensitivity of a particle and its molecular detection sensitivity. This distinction will be addressed in depth in a later section, but as an example, consider the work of Unger et al., in which an AFM tip is used to manoeuvre 60 nm dielectric spheres into close proximity to a gold nanocrescent, has shown in below figure. The LSPR peak shifts by an average of 11 nm when the dielectric sphere is placed in the tip region where the electric field is enhanced. [36] To design optimal particles for biosensing applications, therefore, the particle shape is more important than its effect on the bulk refractive index sensitivity might seemingly indicate.

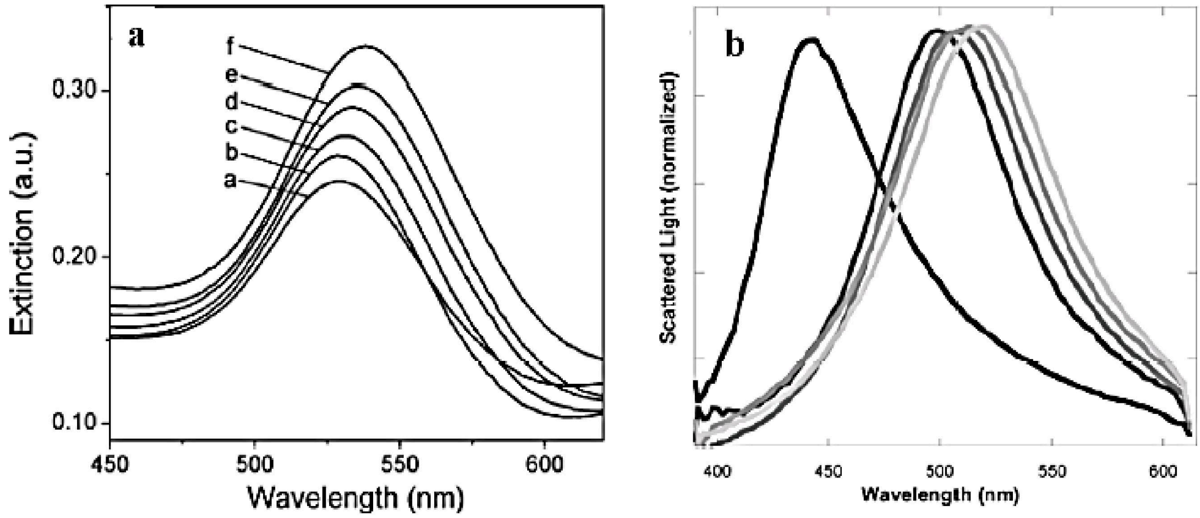


Picture 2: Effect of Shape of Nanoparticles
(a) LSPR shift of gold nanocrescents upon attachment of a 60 nmdielectric sphere.
(b) E-field enhancement of a gold nanocrescent, showing regions of high enhancement at the sharp tips.

Effect of Material of Nanoparticle:

A comparison of gold and silver nanoparticles of similar size and shape highlights this fact. For example, for spheres 50-60 nm in diameter, the refractive index sensitivity is 60 nm/RIU for a plasmon resonance at ~530 nm for gold [33] and 160 nm/RIU for a plasmon resonance at ~435 nm for silver [32].

As another example, for Nano-cubes 30-50 nm in size, the refractive index sensitivity is 83 nm/RIU for a plasmon resonance at 538 nm for gold [37] and 146 nm/RIU for a plasmon resonance at 510 nm for silver [38] has shown in below **Graph 2 and Table 1**.



Graph 2:
Refractive index sensitivity of:
(a) gold nanospheres in the refractive index range n = 1.33-1.50
(b) silver nanospheres in the range n = 1.00-1.56 [39]

Table 1: Effect of Material Composition on LSPR of Particles of Similar Shape and Size: [39]

Particle type	Size (nm)	LSPR Peak (nm)	Sensitivity (nm/RIU)
Gold nanospheres	50	530	60
Silver nanospheres	60	435	160
Gold nanocubes	44	538	83
Silver nanocubes	30	201	146

Chemistry of Melamine:

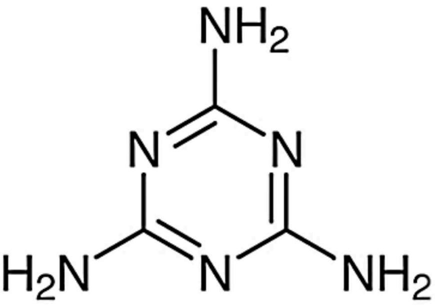


Figure 1: Chemical Structure of Melamine

Melamine or 2,4,6-triamino-1,3,5-triazine is a nitrogen-rich compound and has been widely used in industry for producing melamine-formaldehyde resin. It is white, tasteless and odourless compound. It is a small polar molecule with a molecular weight of 126.12 g per mole. The nitrogen atoms constitute 66.67% of the molecular weight of melamine. It is slightly soluble in water at 20°C (~3.1 g/l). One of the remarkable characteristics of melamine is formation of crystals due to its extensive multiple hydrogen bonding. The structure of melamine, with its three N atoms in ring and three amino groups, enables the melamine to act as both a donor and acceptor in formation of hydrogen bonds, which enable its molecule to form extensive hydrogen bonds with cyanuric acid and biomolecules such as uracil, riboflavin, barbituric acid and uric acid. [40] Here, **Figure 1** shows the chemical structure of Melamine.

Such a substance forms a driving force of the adulteration of milk products with melamine for obtaining high protein content readings. This is also because the conventional Kjeldahl or Dumas test is a method of analysing total nitrogen content, without identifying its sources. It gives an appearance of protein content, not true protein content, and therefore cannot distinguish nitrogen of melamine from other proteins. Although melamine is not inherently a carcinogen, it has low oral acute toxicity. [41]

Overview of Melamine contamination in milk and associated health hazards:

Toxicity data mainly come from studies in sheep, cat, dog, mice, and rat. Toxicity can be classified as acute or chronic. The most common toxicity is renal toxicity, which is also the area of most concern to nephrologists. High-dose ingestion of melamine or chronic administration can induce urinary calculi and acute renal failure and even results in death, especially in babies and children. Studies on toxicity caused by oral ingestion of melamine in humans are non-existent. LD_{50}, the lethal dose of a given compound resulting in death of 50% of tested animals, is 3.1 g/kg of body mass for melamine in rats. In the pig, the melamine plasma half-life is approximately 4 h. In general, melamine and cyanuric acid are not acute toxic because of their large lethal doses. The LD50, the toxic dose, of melamine in rats was 3296 mg/kg body weight and 7940 mg/kg bogy weight for cyanuric acid. Oral ingestion affects the digestive tract, presenting as nausea, vomiting, and diarrhoea**.** [40]

The first case of melamine adulteration was detected in fish meals from Italy during the late 1970s and potato meal for protein was found adulterated with melamine in Germany. Three large scale incidences of using melamine as an adulterant in food and related ingredient occurred during last decade. The first incidence was in South-eastern Asian countries in which large outbreaks of nephrotoxic renal failure, occurred in dogs and cats during late 2003 and 2004, later on its cause correlated melamine contaminated pet food. In early 2007 very large outbreak of pet food–associated nephrotoxic renal failure and deaths of thousands of cats and dogs occurred in the North America. Veterinary scientists attributed the pet deaths to the nephropathy induced by crystals containing melamine and cyanuric acid present in the kidney. Another outbreak occurred in 2008, this time victim were infants and young children. Thousands of infants were rushed to hospital with symptoms of crying and internal bleeding. Again melamine was found as culprit. The Chinese government reported that 294000 babies had fallen ill, 51900 were hospitalized and 6 died, after consuming melamine-tainted infant formula. The children in other parts of Asia such as Taiwan, Singapore and Vietnam were also affected. [40]

The World Health Organization (WHO) in September 2008 reported toxicity, preliminary risk assessment and guidance on levels of melamine in food. In collaboration with the Food and Agriculture Organization (FAO) supported by Health Canada, WHO conducted an expert meeting to review toxicological aspects of melamine and cyanuric acid in December 2008. The Codex Alimentarius Committee (CAC) has promulgated the maximum limits of 1 mg/kg in powdered infant formula and 2.5 mg/kg in food (other than infant formula) and feed in 2010, and 0.15 mg/kg for melamine in liquid infant formula finally adopted in 2012.The melamine-contamination incidents prompted the US Food and Drug Administration (FDA), the European Community (EC) and other countries and regions to establish the criteria of Maximum Residue Limits (MRLs) for melamine in various food products used every day. The most common melamine limits established by different countries were 1 mg/kg for infant formula and 2.5 mg/kg for other milk and milk-based foods or all other foods. [40]

The FSSAI has added a new regulation on melamine in milk and milk products through its notification dated 5th January, 2016. According to the amendment a new section has been added to the regulation called „Other contaminants‟ in the Food Safety and Standards (Contaminants, toxins and Residues) Regulations, 2011. The new amended regulations will now be called the Food Safety and Standards (Contaminants, toxins and Residues) Amendment Regulations, 2016.The maximum limits of melamine content are 0.15 mg/kg in liquid infant formula, 1 mg/kg in powdered infant formula and 2.5 mg/kg in other food. [40]

Importance of rapid and reliable melamine detection methods in milk:

The detection of melamine in food products has been the subject of much recent research. Common methodologies used for melamine detection are mainly based on high performance liquid chromatography (HPLC), LC–mass spectrometry (MS), gas chromatography, enzyme-linked immunosorbent assay (ELISA) and capillary electrophoresis. Although the above methods are generally very sensitive and accurate, they are not suitable for routine analysis because of such drawbacks as expensive equipment, complicated and time-consuming procedures for sample pre-treatment, specific skills for operation.

Recently, various methods including electrochemical, surface-enhanced Raman spectroscopy (SERS), localised surface-plasmon resonance (LSPR), infrared spectroscopy and colorimetric sensor based on polydiacetylene (PDA) liposomes, have been investigated to detect melamine, some of which suffer from complicated chemical synthesis, high cost or poor sensitivity. Therefore, there is still an urgent need to develop a rapid, sensitive and facile method for the efficient detection of melamine in food.

In recent years, significant efforts have been made on the development of nanoparticles (NPs)-based assay for the fast and effective detection of melamine. For example, Au and Ag NPs modified with different stabilisers have been used as colorimetric probes for melamine sensing in liquid milk and infant formula, through the hydrogen bonding recognition, electrostatic interaction, or electron donor–acceptor interaction between melamine and the stabiliser at the nanoparticle interface. [42]

Another studies shows that, when the nanoparticles approach each other and aggregate, the colour of the silver nanoparticles changes from light yellow to dark yellow (straw yellow), due to the shift of the surface plasmon band to longer wavelength. The major advantage of metallic nanoparticles-based assays is that molecular recognition event can be transformed into colour change, which can be easily observed by the naked eyes, and therefore sophisticated instruments are not required. In this study, a simple, economical and field-portable visual method, less time required than conventional method for the detection of melamine was developed. [43]

Spectrophotometric Method for using Melamine Detection Quantitatively:

The proposed method can be used for the detection of melamine in raw milk by monitoring with the naked eyes and UV-Vis spectroscopy at room temperature. The whole detection process could be completed within 5hour after adding Nanoparticles to the sample with minimal pre-processing. Reason behind using spectroscopy methods for detecting of melamine in milk over traditional techniques are having Speed and Real-Time Monitoring which is particularly important in the food industry, where quick quality control and safety assessments are crucial. Traditional techniques, on the other hand, typically requires longer analysis times. Minimal Sample Preparation, Portability, Cost-Effectiveness etc. are also another reasons or using this method. While Traditional techniques remains a powerful analytical technique with high accuracy and precision, spectroscopic methods like LSPR offer unique advantages in terms of speed, ease of use, and real-time monitoring, making them well-suited for rapid and on-site detection of melamine in milk and other food safety applications. These factors contribute to their increasing adoption in the food industry for quality control and safety assurance.

Rationale for utilizing green-synthesized Ag-NPs for melamine detection in milk:

Green synthesis methods use plant extracts or other natural materials as reducing and stabilizing agents. This approach is environmentally friendly, as it reduces the need for hazardous chemicals and minimizes the generation of toxic waste compared to traditional chemical synthesis methods. Green-synthesized Ag-NPs are often biocompatible and pose minimal risks to human health. This is particularly important when developing sensors for food safety applications, as any potential contamination of food samples by the nanoparticles is kept to a minimum. Coming to the point of Sustainability, the Utilizing renewable and natural resources for nanoparticle synthesis aligns with sustainability goals. It reduces the environmental impact associated with conventional chemical synthesis methods and promotes the responsible use of resources.

In various studies, rapid sensing of melamine in milk by green synthesized Ag-NPs were reported with a LOD [limits of detection] of 0.04 mg/L and 0.1 ppm, respectively. $AgNO_3$ was used as a precursor and leaf extract as a reducing agent for green synthesis of Ag-NPs in the mentioned research. Dispersed Ag-NPs give strong absorption in UV–Vis spectra around 410 nm. On the other hand, the exocyclic amino groups of melamine (positive charge) attach to these reduced surface of Ag-NPs (negative charge). Thereby, hydrogen bonding between Ag NPs and melamine molecules cause aggregation of Ag-NPs which produce visual colour changes with appearance of new absorption. More and more Ag-NPs aggregate as the concentration of melamine increases and the absorption spectra. The green synthesis of Ag-NPs with leaf extracts with respect to chemical synthesis, provides good yield results for the single-stage melamine detection. In general, melamine detection with Ag-NPs is performed in two steps. The first step requires synthesis and functionalization of Ag-NPs, and the second step involves the detection of melamine using functionalized Ag-NPs. Green synthesis of Ag-NPs is not only an eco-friendly process but also a rapid and one-step method for the analyte

detection. In this strategy, the analyte interferes with the biosynthesis of the NPs and then is detected by the NPs. [44]

Aims & Objective of this Experiment:

The overarching aim of the research project titled "Green Synthesis of Silver nanoparticles and Colorimetric Detection of Adulterated Melamine in Milk based on LSPR of green synthesized Silver Nanoparticles" is to develop a sustainable and environmentally friendly approach to both the synthesis of silver nanoparticles (AgNPs) and the detection of melamine, a harmful food adulterant, in milk. The primary objectives include optimizing the green synthesis of AgNPs using natural sources, characterizing the synthesized nanoparticles, and functionalizing their surfaces for enhanced specificity. Furthermore, the project seeks to design a robust colorimetric detection method based on the Localized Surface Plasmon Resonance (LSPR) properties of the green-synthesized AgNPs, with a focus on achieving high sensitivity and selectivity for melamine detection. Real-sample validation and comparison with existing methods will be conducted to assess the practical applicability of the developed technique. Additionally, the project aims to evaluate the environmental and health impact of the entire process, aligning with sustainability and safety considerations in food safety research.

Chapter 2:

Materials & Methods

Materials & Methods

Reagents & Materials:

Melamine was purchased from Loba Chemie PVT. LTD, India, and Silver nitrate [$AgNO_3$], sodium citrate [$Na_3C_6H_5O_7$], sodium borohydride [$NaBH_4$], sodium bicarbonate [$NaHCO_3$], Trichloroacetic acid [TCA] and chloroform[$CHCl_3$] are purchased from Forbes Chemical and used in this method. ***Swietenia macrophylla*** leaf extract is used in order to synthesis eco-friendly AgNPs. All solvents and reagents are of analytical grade and were used without further purification. Doubly-distilled water was used throughout the experiments. The raw milk was purchased from the local pasture. It's important to note that, the specific roles and concentrations of these reagents and materials, as well as the detailed protocol for the method, has explained in the experimental procedure to effectively detect melamine in milk using green-synthesized AgNPs. Proper handling and disposal procedures for chemicals and waste should also be followed in accordance with safety guidelines and regulations.

Apparatus:

Spectra absorption data were acquired at room temperature using Lasany Microprocessor UV-Vis Double Beam Spectrophotometer with variable Banwidth. pH values were determined with a Systonic S-906/ Microprocessor pH meter, while centrifugation procedures were conducted using a REMI Cooling Centrifuge. Solubility tests were examined in a Hot Air Oven (Bhattacharya & Co., India) and BOD Incubator Shaker (G.B Enterprise, India). Material weights were measured using an Internal Digital Mettler Toledo ME Precision Balance Machine with accuracy 0.01gm. Remi 2-MLH Magnetic Stirrer & Hot plates are also used. Remi CM 101 vortex is also used for mixing solutions properly. Additionally, various glass laboratory apparatus such as mortar and pestle, measuring cylinders, test tubes, Petri dishes, centrifuge vials, pipettes, and volumetric flasks were employed in the experiments.

Synthesis of Silver Nano-particles:

The synthesis of silver nanoparticles (AgNPs) from green leaf extract involves a green environmentally friendly and simple method that utilizes natural compounds present in the leaf extract as reducing and stabilizing agents. Here's a simplified step-by-step guide on how this synthesis process typically works:

a. Preparation of Leaf Extract:

In this research, silver nanoparticles are synthesized using leaf extract derived from the ***Swietenia macrophylla*** tree, commonly known as the mahogany tree. The leaf extract from this particular tree is rich in potent reducing substances such as various polyphenols, limonoids, essential oils etc., which prompted its selection as the preferred source for silver nanoparticle synthesis in this study. [49] Here **Figure 1** shows the process of preparation of leaf extract.

Figure 1: Process of Preparation of Leaf Extract

Eco-Friendly Synthesis of Silver Nano-Particles:

Figure 2 demonstrate the process of preparation of Silver nanoparticles from leaf of ***Swietenia macrophylla*** tree step by step.

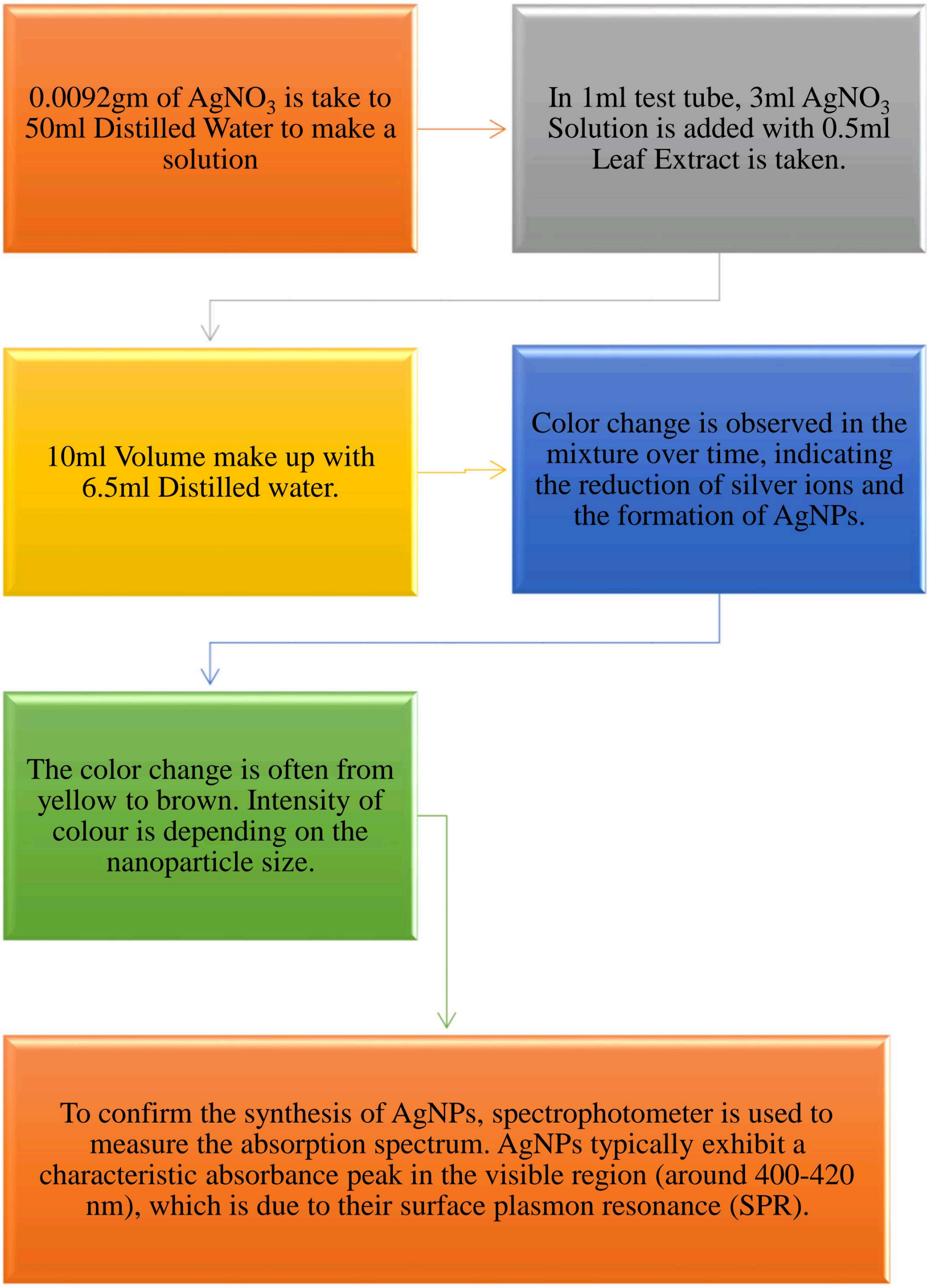


Figure 2: Process of eco-friendly Synthesis of AgNPs from Leaf Extract

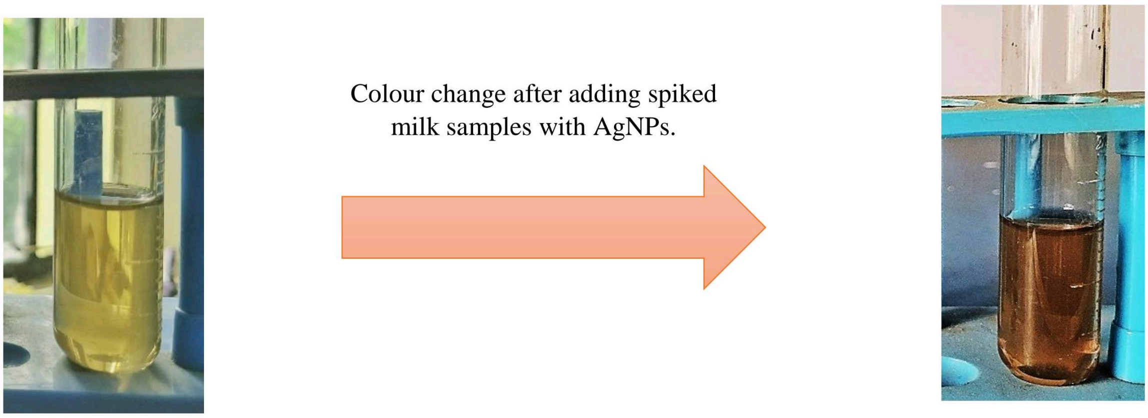
Colorimetric Detection of Melamine in Liquid Milk:

A typical colorimetric analysis for melamine detection in raw milk was conducted as follows:

Initially, 10 mL of raw adulterated liquid milk was placed in a 15 mL centrifuge tube. Subsequently, 2.0 mL of a 10% mixed solution containing Trichloroacetic acid [TCA] and chloroform[$CHCl_3$] was added to the tube. The mixture was vigorously vortexed for 1 minute to precipitate proteins within the sample matrix. Following this step, the mixture was sonicated at a temperature of 20°C for a duration of 15 minutes. After sonication, the sample was centrifuged at 6500 rpm for 10 minutes, facilitating the separation of the precipitated components.

In the next stage, the supernatant was carefully transferred to a separate centrifuge tube and the pH was adjusted to 8.0 by the addition of a 10% $NaHCO_3$ solution. A further centrifugation step at 6000 rpm for 10 minutes was performed to eliminate any remaining deposits, resulting in a refined solution suitable for subsequent detection.

Subsequently, 3mL of this refined clear solution was mixed with 3 mL of a eco-friendly synthesised AgNPs solution. The mixed solution was allowed to react at room temperature for a period of 20 minutes. Finally, the absorption spectrum of the reacted solution was recorded using a cuvette with a 1 cm path length. The concentration of melamine was quantified based on the absorption ratio (A_{700}/A_{300}) or assessed through visual observation by the naked eye. The colour transformation of the solution was also documented using a digital camera shown in **Picture 1**.



Before

After

Picture 1: Shows the colour change of AgNPs after adding Spiked Milk Sample

Chapter 3:

Results & Discussion

Results & Discussion

Characterization of Eco-Friendly Synthesized Ag-NPs using UV-Vis Spectroscopy:

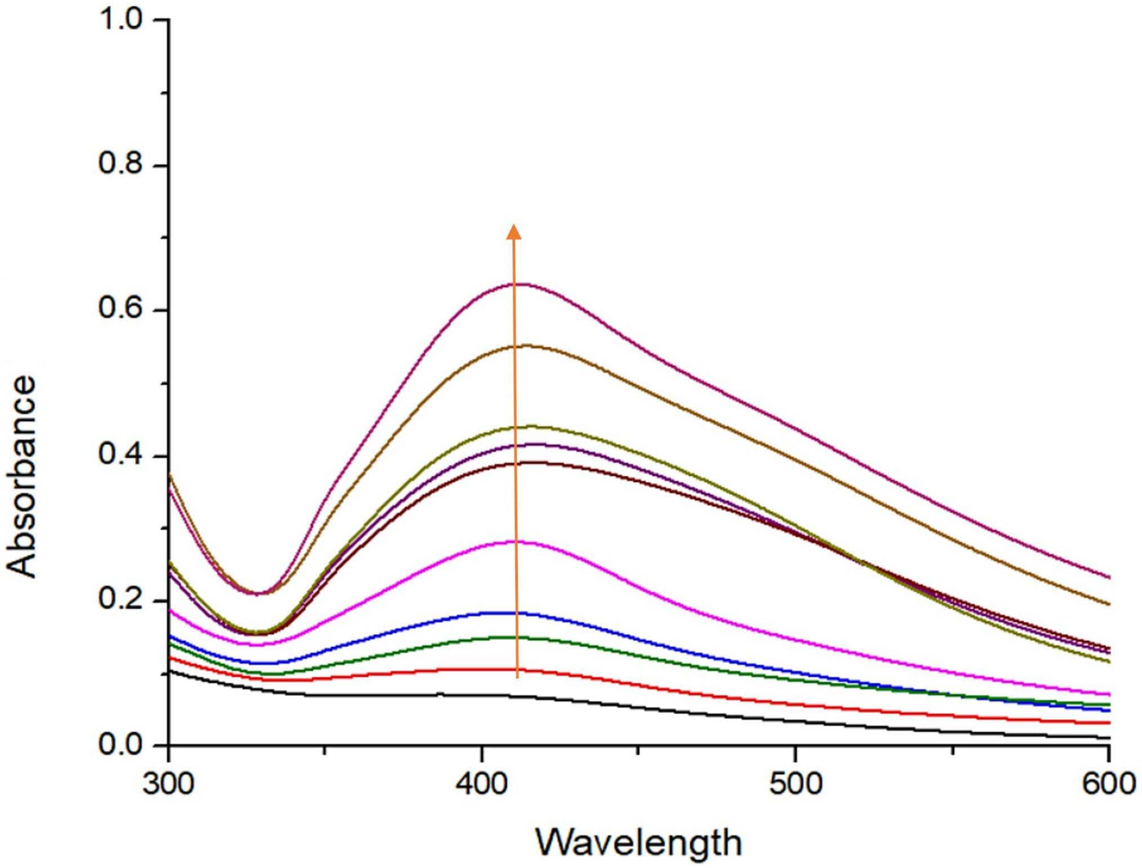
The absorption peaks of gold and silver nanoparticles are closely related with space between nanoparticles, when the state of nanoparticles changes from dispersive to aggregated, the absorption peaks of nanoparticles shift obviously with a corresponding change of absorption strength. [48]

The absorption spectrum of stable Ag NPs shows a typical surface plasmon resonance absorption peak at 400 nm around, demonstrating Ag NPs are well dispersed. After addition of melamine into Ag NPs solution, the Ag NPs quickly aggregate together, and the solution colour changes from yellow to straw yellow or brown as shown with the insert photographs Which is Shown in **Picture 1**.

⇨ Concentration of AgNPs increased

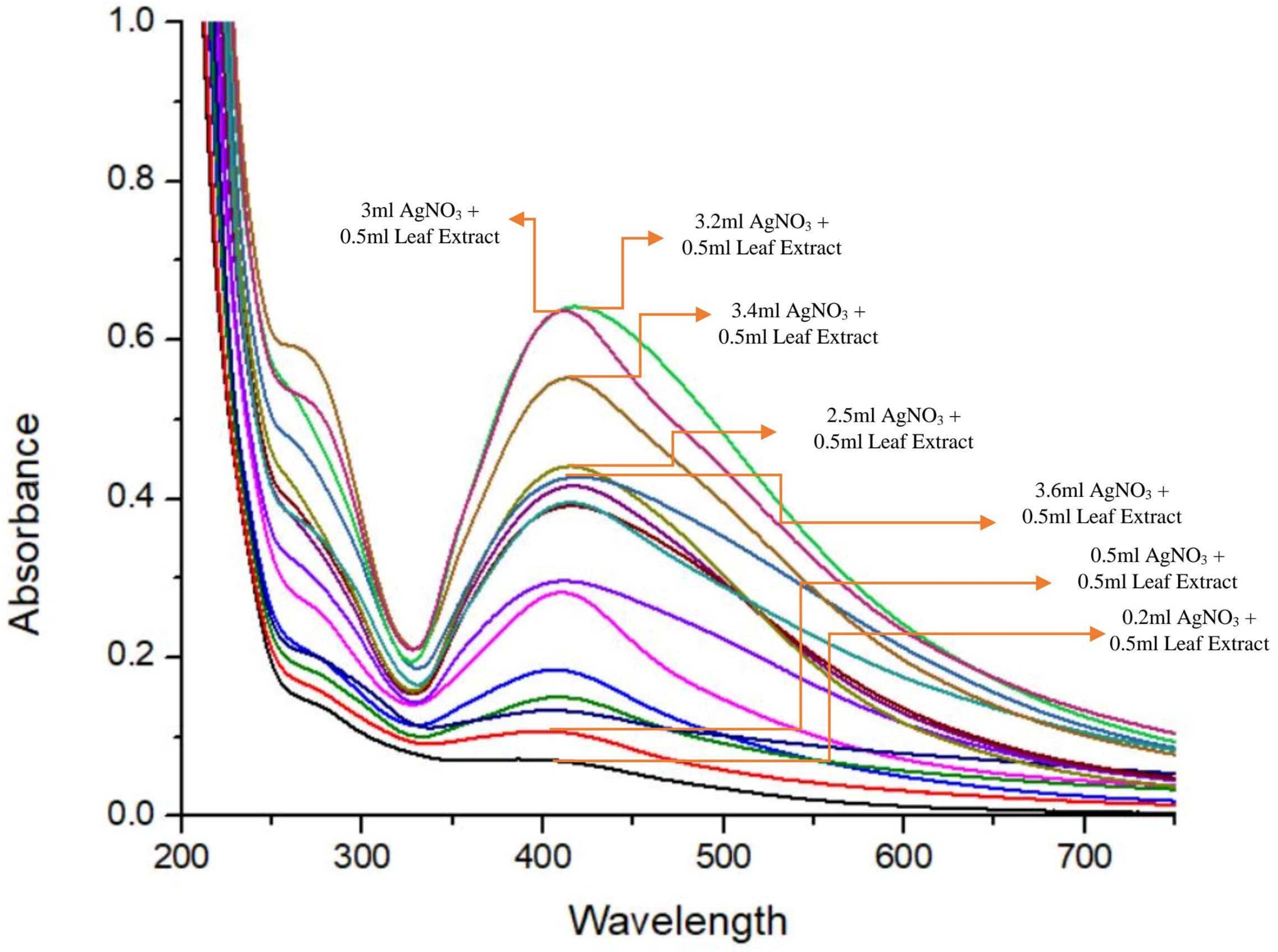
Picture 1: Shows the colour change of AgNPs from lighter to deep colour as increasing the concentration of $AgNO_3$ addition with constant concentration of Leaf Extract

Based on the **Graph 1**, it is confirmed that there is a direct correlation between the concentration of $AgNO_3$ and the absorption ratio. Additionally, variations in the concentration of $AgNO_3$ result in observable changes in coloration, which are influenced by the size and shape of the nanoparticles. These colour changes are typically observed within a span of three hours at room temperature. However, when heat is applied, approximately at 45°C, after adding the leaf extract to $AgNO_3$, the colour changes become evident within a shorter timeframe, around 30-45 minutes. This accelerated colour change, discernible to the naked eye, offers a highly effective means for detecting melamine in milk samples.



Graph 1: Shows the direct correlation between the concentration of $AgNO_3$ and the absorption, by increasing the concentration of $AgNO_3$ the absorption peak will also increase.

In the **Graph 2**, the relationship between the concentration of $AgNO_3$ solution and the absorption ratio is illustrated. The experiment involved keeping the leaf extract constant at 0.5ml and making up the total volume to 10ml with distilled water. As the concentration of $AgNO_3$ solution increased from 0.2ml to 3.6ml, there was a noticeable increase in the absorption ratio within the range of 400nm to 430nm. However, beyond 3.2ml of $AgNO_3$ solution, the absorption ratio began to steadily decrease. As a result, the determined optimal concentration of $AgNO_3$ solution for a particular leaf extract concentration is 3ml in total volume of 10ml solution.

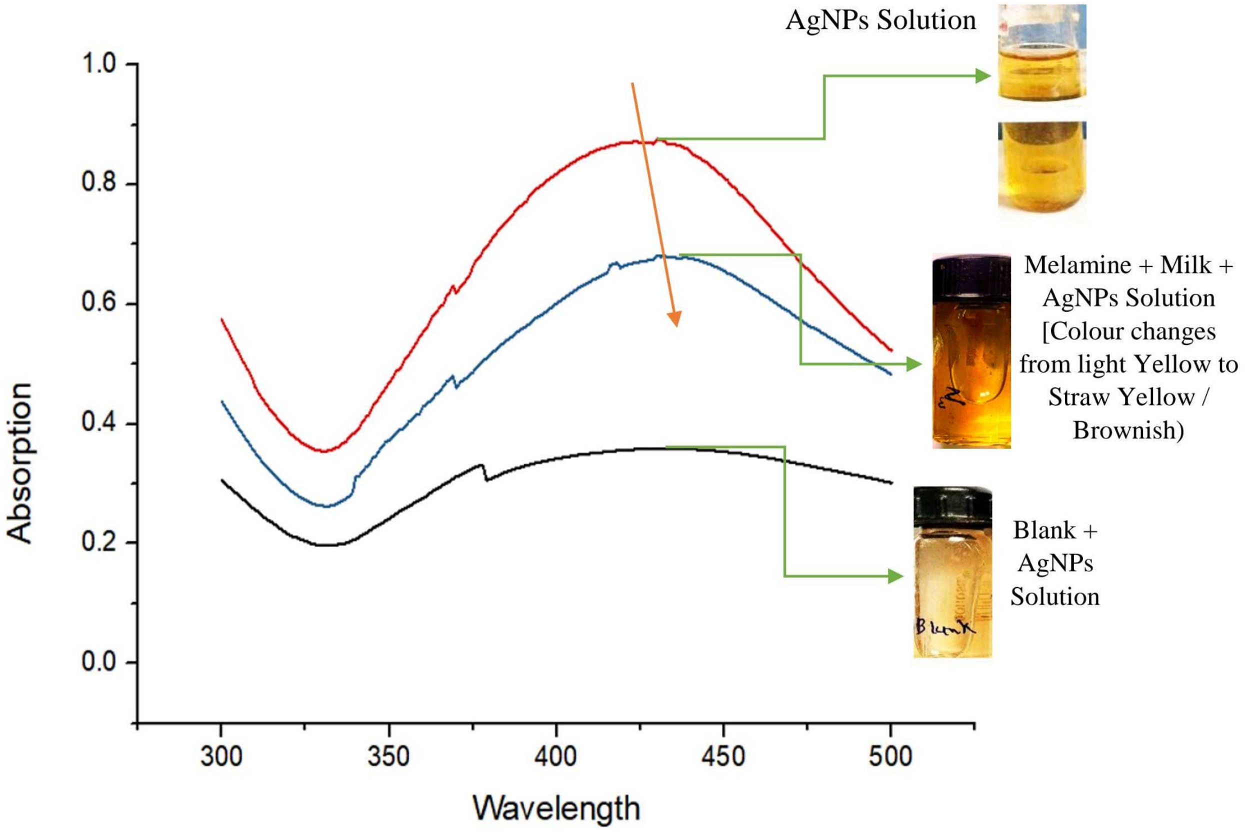


Graph 2: Shows the relationship between the concentration of $AgNO_3$ solution and the absorption

Detection principle and interaction between Ag-NPs and melamine:

Green synthesized Ag NPs can be stabilized in aqueous solution by coating it with negatively-charged ions, and electrostatic force counteract the effects of Van der Waals' force between molecules, with the result of homo-disperse of Ag NPs. Melamine molecule is a small molecule which contains three exocyclic amino groups ($-NH_2$) and a three-nitrogen hybrid ring. The negatively-charged ions can attach each other with positively charged exocyclic amino groups ($-NH_2$), with the result that melamine molecules are attached to the surface of Ag NPs.

In our experiments, when melamine was added into Ag NPs solution, the Ag NPs aggregated together, simultaneously, the colour of Ag NPs solution changed with spectral variations as shown in **Graph 3**. The aggregation of Ag NPs might be induced either by the three exocyclic amino groups ($-NH_2$) or by the three-nitrogen hybrid ring.



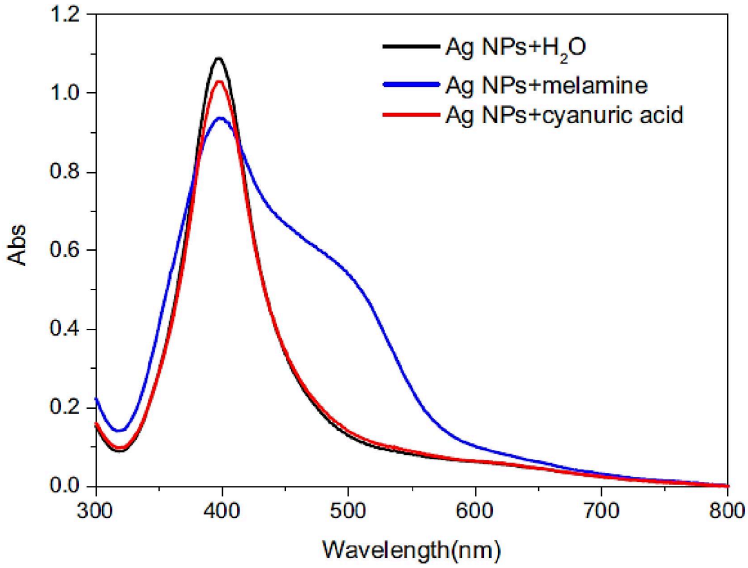
Graph 3: Shows the colour change of AgNPs after adding spiked milk sample & also shows the decrease of absorption of spiked sample.

Graph 3 shows that, in the presence of melamine, the absorption at 416 nm of the original Ag NPs decreases and shows a slight shift, by which we can conclude the simple, rapid and field-portable colorimetric method can be developed for the analysis of melamine by naked eyes or UV-Vis spectroscopy.

Upon the addition of a spiked milk sample to the AgNPs solution, a noticeable transformation occurs as the vibrant yellow colour of the mixture shifts towards a distinctive straw yellow or even a brown hue. This alteration in colour serves as a label-free indicator, signifying the potential presence of melamine. What's particularly striking is the discernible disparity observed when comparing the sample containing melamine to the blank, highlighting a substantial difference between the two. In addition to the visual color change, another significant cue is provided by the reduction in the absorption peak of the silver nanoparticles synthesized from the leaf extract. This further reinforces the evidence of melamine's presence within the milk sample, underlining the sensitivity and effectiveness of this detection method.

In order to explore the real reason of Ag NPs aggregation, in different studies, carried out the control experiment using cyanuric acid as a substitute of melamine. [46] Melamine is easily hydrolysed to produce cyanuric acid. In the molecular structure of cyanuric acid, three hydroxy groups (-OH) replace the three amine groups ($-NH_2$) of melamine, and the three-nitrogen hybrid ring still remains.

The experimental results showed in **Graph 4**[46] that, cyanuric acid could not induce the aggregation of Ag NPs, as shown in previous figure. It is obvious that only three amino groups of melamine are key groups for the interaction between melamine and Ag NPs. The three amino groups caused a rapid yellow-to-red colour



Graph 4: Absorption spectra of Ag NPs in presence of water (black line), cyanuric acid (red line) and melamine (blue line). [46]

change, whereas, the three-nitrogen hybrid ring is irresponsible for the interaction between melamine and Ag NPs.

On the other hand, every melamine has six equivalent sites which are to form double NH—NH bonds with a similar site of another melamine molecule. [47] This is the cause of melamine-coated Ag NPs could be cross-linked by NH—NH bonds between melamine molecules as shown schematically here. Thereby, the aggregation of Ag NPs could be induced. **Figure 1** and **Picture 2** depicts the mechanism for colorimetric detection of melamine by label-free AgNPs. [46][47]

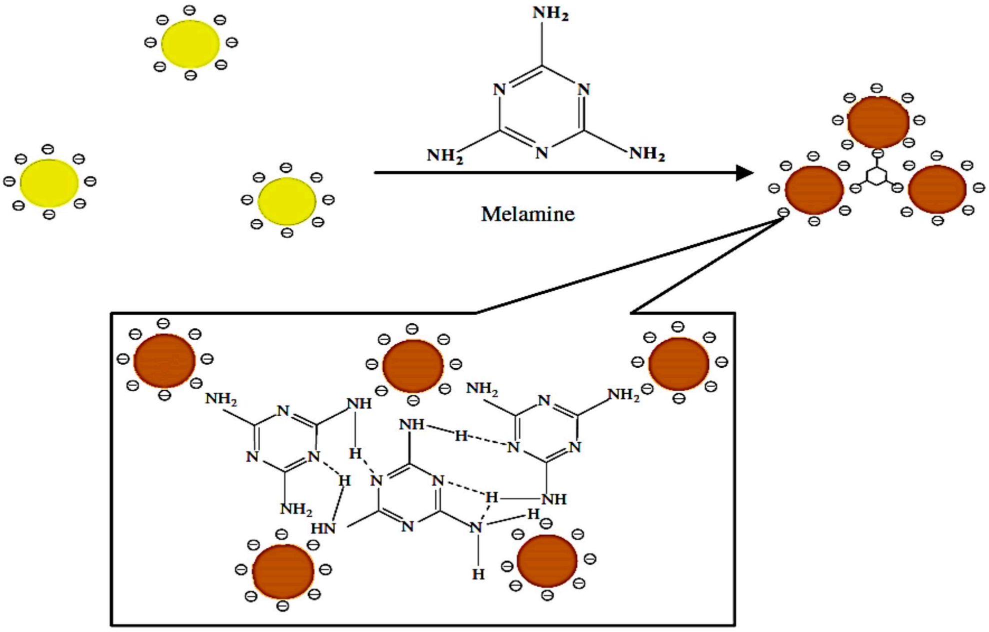
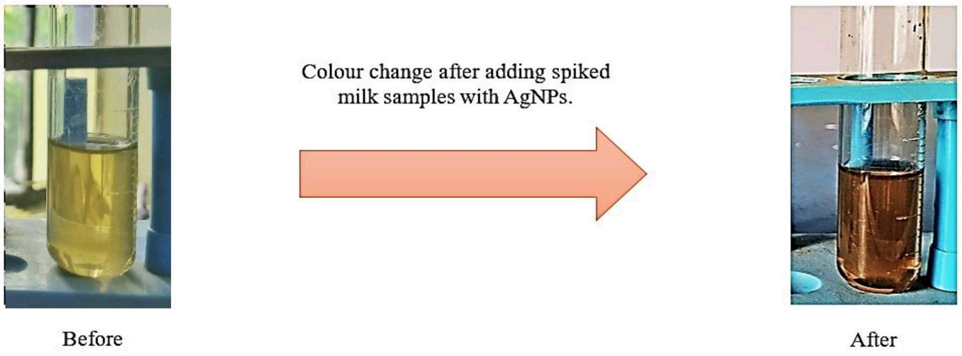


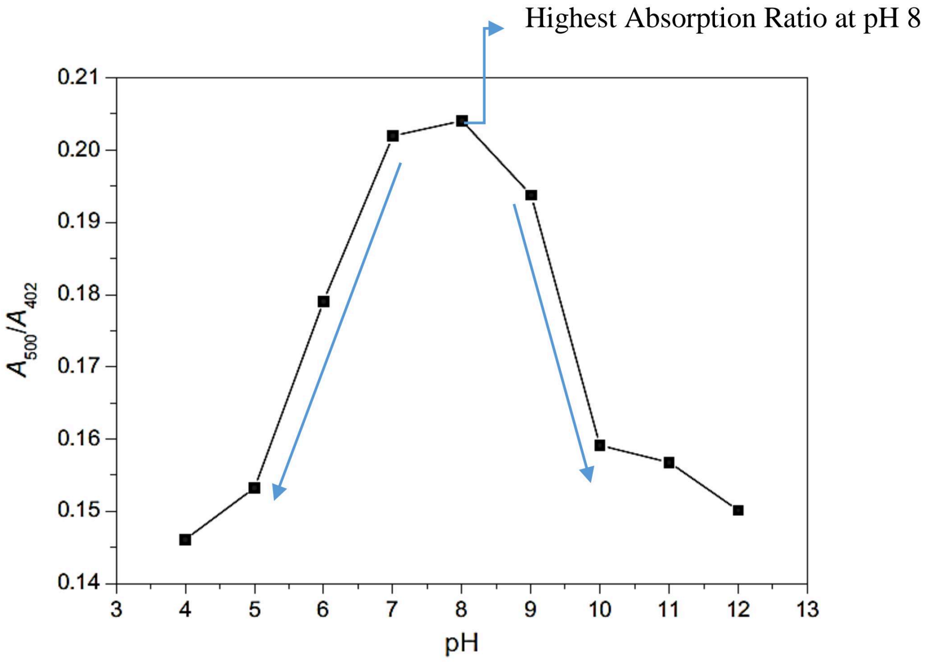
Figure 1: Schematic representation of the Ag NPs colorimetric mechanism for melamine detection [46]



Picture 2: Colour change due to Colorimetric sensing capacity of Green Synthesized Nanoparticles

Effects of Media pH & time on the Absorption Ratio:

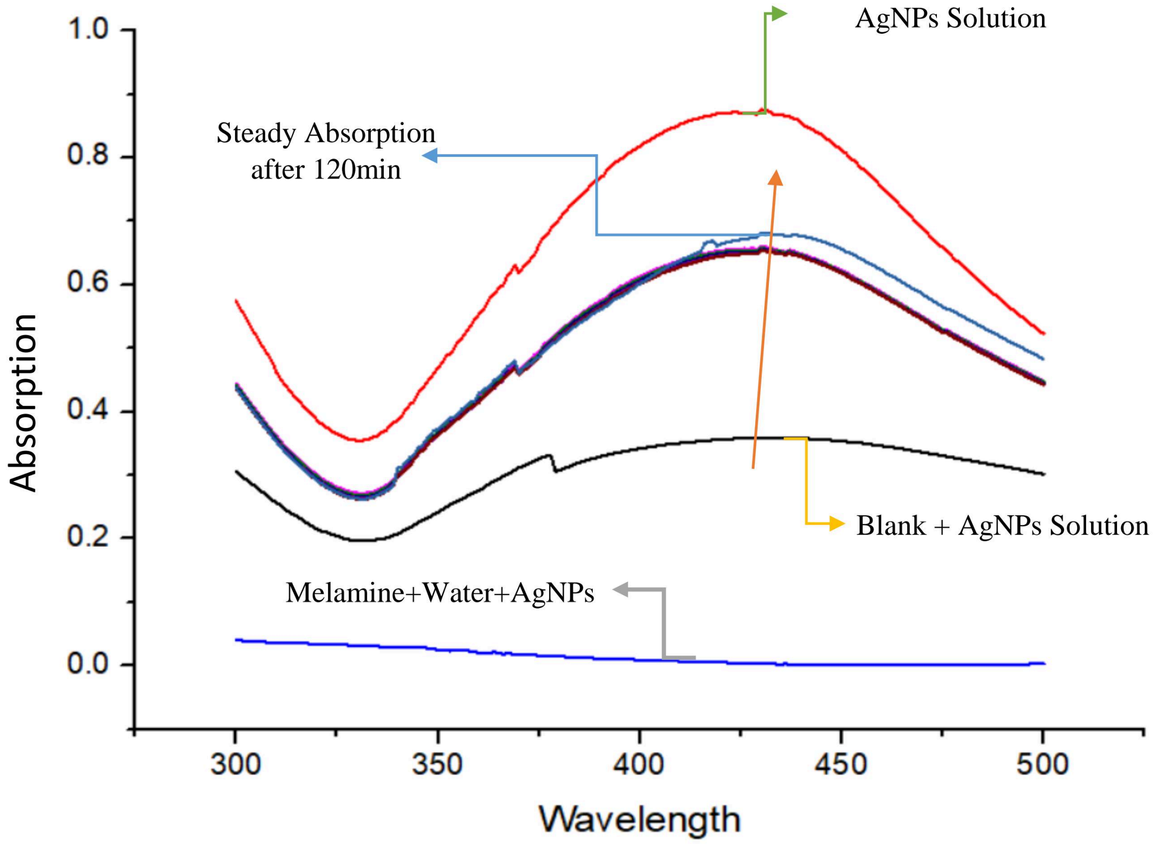
The interaction of Ag NPs and small molecules (melamine) can be affected by media pH. Melamine is a weak base with pKa of 5.05. Media pH can also affect the form of melamine in aqueous solution. The effect of media pH in the range from 4.0 to 12.0. Media pH of Ag NPs was adjusted with hydrochloric acid or sodium hydroxide. To take 0.1 mM melamine for the example, the profile of absorption ratio (A_{500}/A_{402}) versus media pH was obtained, as shown in **Graph 5**. [46]


Highest Absorption Ratio at pH 8

Graph 5: Effects of media pH on the absorption [46]

It can be seen that in acidic media (pH < 5.0) and basic media (pH > 10.0), the absorption ratio (A_{500}/A_{402}) is lower; at pH 7.0~9.0, the highest absorption ratio (A_{500}/A_{402}) has been obtained. It is probably due to the fact that melamine was hydrolysed at pH < 5.0 and pH > 10.0, amino groups were gradually replaced by hydroxyl groups, and melamine was finally transformed into cyanuric acid that could not induce the aggregation of Ag NPs. [45] The other reason is that Ag NPs partially aggregate, and could not interact with melamine in the media with relatively low pH (pH < 4.0) or relatively high pH (pH > 12.0) which can be confirmed by control experiments. Thus, pH of media was chosen as 8.0.

The reaction time between Ag NPs and melamine is a key point that affects colorimetric assays, and the relationship between reaction time and absorption ratio (A_{500}/A_{300}) was investigated by adding 3 mL of 1.0 mM melamine adulterated milk solution into 3 mL Ag NPs solution, as shown in **Graph 6**.



Graph 6: Effects of Time on the absorption.

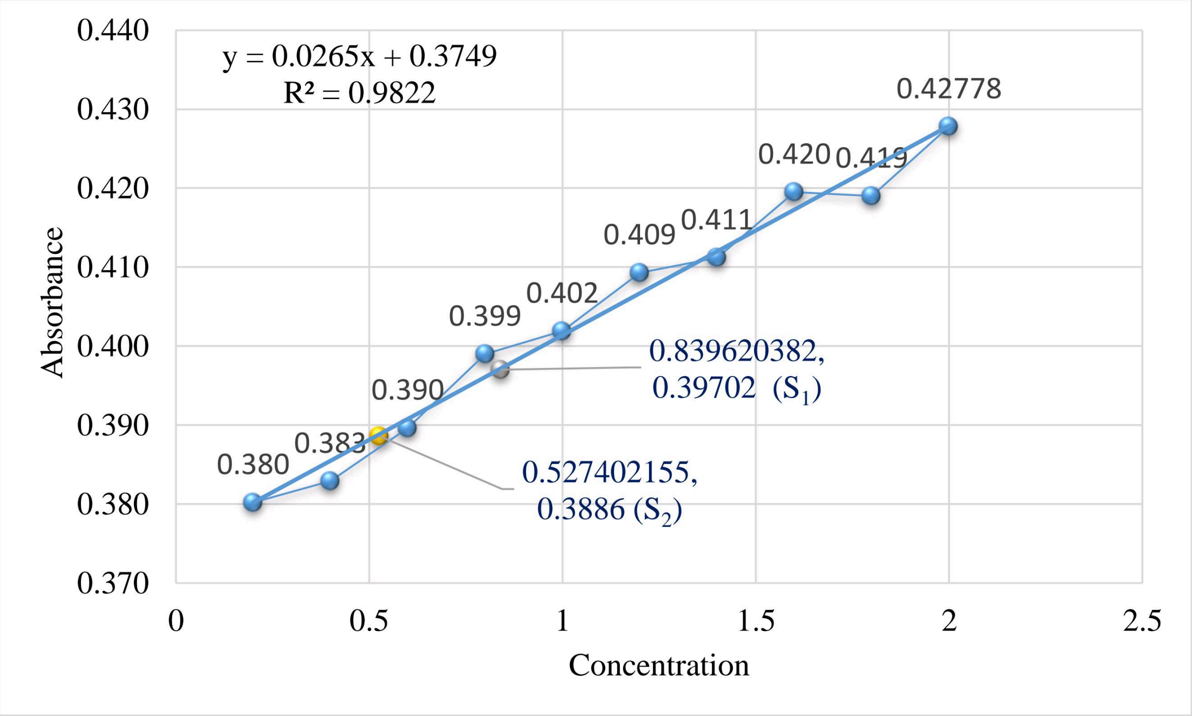
The absorption profile exhibited a gradual increase from 1 minute to 120 minutes, with the values remaining relatively stable between the 90 to 120-minute mark. This trend suggests that the aggregation of Ag NPs approached completion during this time frame. As a result, the optimal detection time was selected to be approximately 120 minutes for the process.

Construction of a calibration curve and analysis of unknown melamine concentration in milk:

Standard melamine solutions were prepared by dissolving the known concentration of melamine in milk. AgNPs were used to detect the melamine in standard melamine solution under the above optimized conditions, and the calibration curve were drawn by plotting the absorbance against the melamine with different concentration. The concentration of samples and its measured absorbance in particular wavelength 425nm is in following **Table 1**.

Table 1: Different concentration of Absorbance of melamine in milk in 425 nm:

Concentration [gm/10ml]	Absorbance	Concentration [gm/10ml]	Absorbance
0.2	0.380	1.2	0.409
0.4	0.383	1.4	0.411
0.6	0.390	1.6	0.420
0.8	0.399	1.8	0.419
1	0.402	2	0.42778



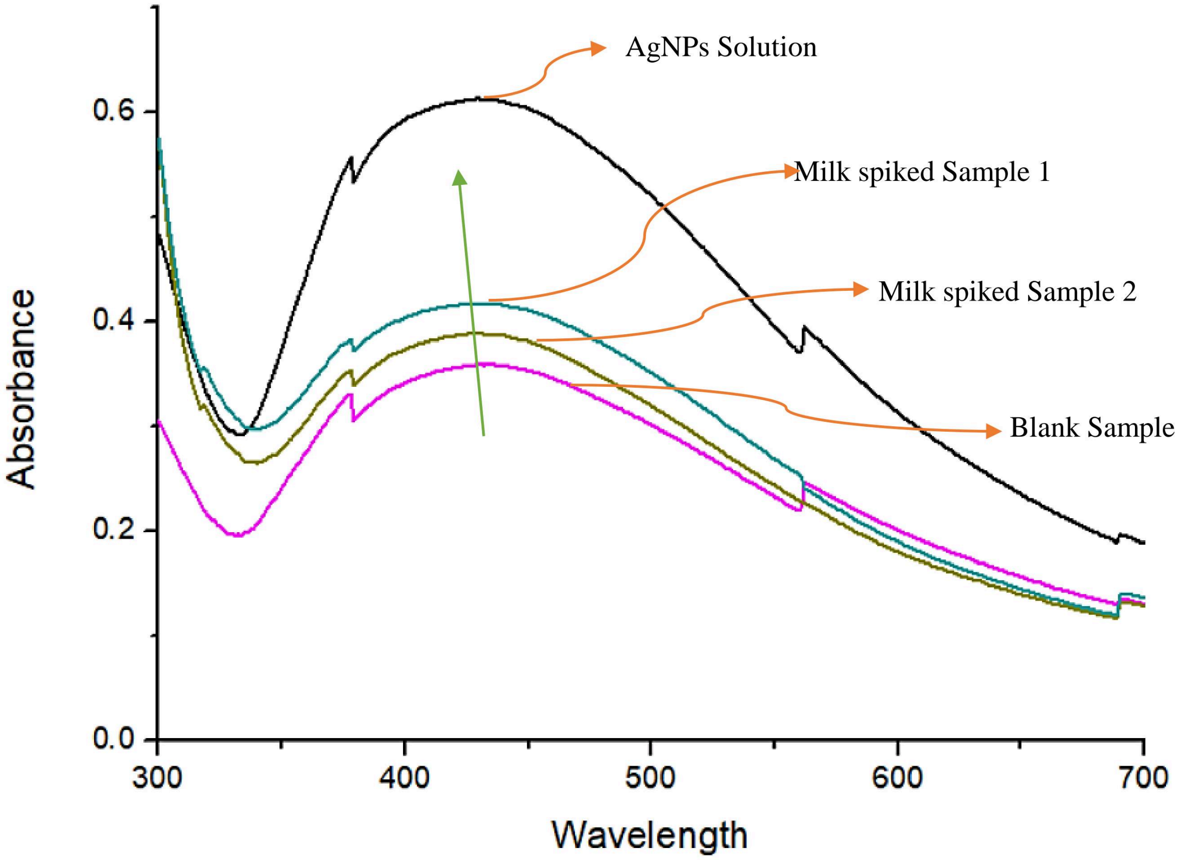
Graph 7: Calibration Curve shows the absorbance of concentration of Melamine at 425nm wavelength

Graph 7 shows the calibration curve was linear over a concentration range of 0.2 to 2 mg/L with the regression equation y = 0.0265x + 0.3749 and R^2 of 0.9822. There was a gradual increase in the absorption value of A_{600}/A_{300} with an increase in the concentration of melamine.

Analysis of melamine in real samples:

To validate the analytical performance of AgNPs to detect melamine in milk samples, two different amounts of melamine were added in two milk samples. The spiked milk samples were extracted and analysed for qualitative and quantitative estimation of melamine under optimized conditions.

Slight colour change of AgNPs was observed on the addition of filtrate sample. The colour changes from yellow to straw yellow/brownish colour on the addition of filtrate from melamine-spiked samples and confirmed by the change in absorption spectra and spectroscopic results are shown in **Graph 8**.



Graph 8: Absorption spectra of Ag NPs in the presence of different concentrations of melamine in real liquid milk samples

The above data were taken after 90 min. The data of unknown concentrations of melamine in milk samples is measured in spectrophotometer, and the peak was shown around 425nm of wavelength, so using the standard curve the concentration of melamine in milk sample is determined and recovery & relative standard deviation is compared in **Table 2**.

Table 2: Data comparison of unknown concentration of liquid milk samples:

Sample Name	Measured Absorbance in 425nm	Calculated Concentration [gm/10ml]	Actual Concentration [gm/10ml]	Recoveries of Melamine (%)	Relative Standard Deviation (n=2)
Sample 1	0.39702	0.839620382	0.85	98.77	0.1561
Sample 2	0.3886	0.527402155	0.57	92.5	0.162

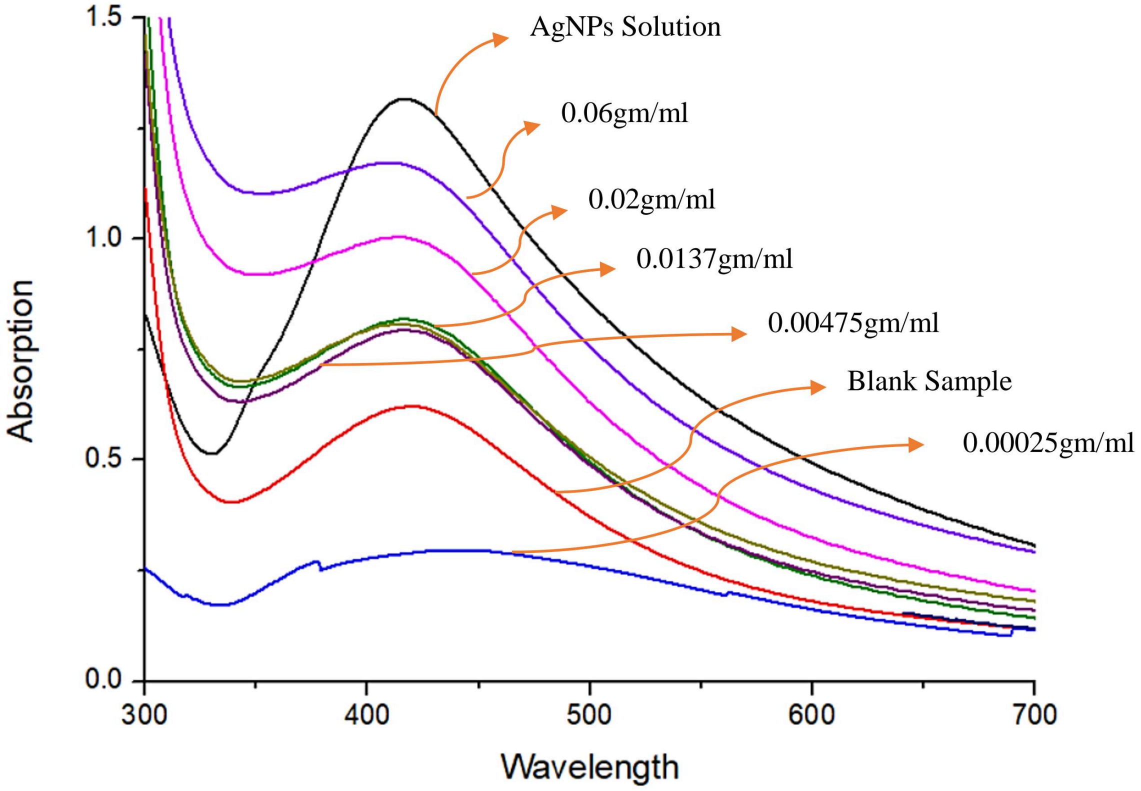
The recoveries of melamine from spiked milk samples varied from 98.77% to 92.5%, with an average recovery ± standard deviation (n = 2) of 95.634 ± 0.59. In general, the method accuracy and precision were good, with recoveries greater than 95%.

Determination of limit and selectivity of Melamine in Liquid Milk Sample with green synthesized Ag-NPs:

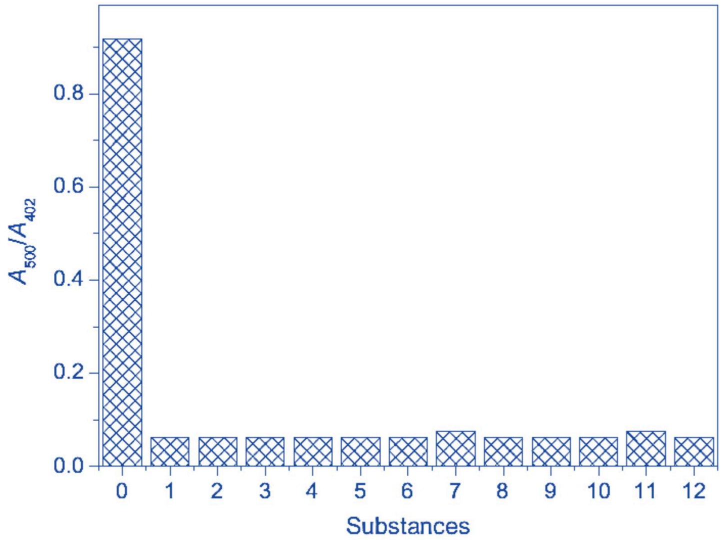
Graph 9 illustrates the absorption curve of spiked milk samples adulterated with varying concentrations of melamine. The method allows for the accurate determination of melamine concentration down to 0.00475 g/ml. This detection limit is established by observing that the absorption curve at a concentration as low as 0.00025 g/ml exhibits a linear peak and falls below that of the blank solution. Therefore, we can confidently assert that this analytical method enables the measurement of melamine concentrations down to 0.00475 g/ml.

[46] In order to explore the specific detection of melamine in raw milk using AgNPs, some specific study shows that the interference of common ions and excipients in raw milk for determination of 0.005gm/ml melamine. With the tolerable concentration ratios for

interference at the ±7% level, the results for AgNPs solution after adding different substances are shown in **Graph 10**.



Graph 9: Shows the detection limit of melamine in real milk samples



Graph 10: Shows the selectivity of Melamine with AgNPs at 420nm-430nm with respect to other substances present in Milk [46]
Here in this graph the Substances: 0, melamine; 1, vitamin C; 2, lactose; 3, glucose; 4, polypeptide; 5, NH^{4+}; 6, Na^+; 7, K^+; 8, Ca^{2+}; 9, SO_4^{2-}; 10, Cl^-; 11, NO_3^-; 12, Cl^-

Chapter 4:
Conclusion & Reference

Conclusion

In summary, this research presents a robust and straightforward colorimetric assay designed to detect melamine in liquid milk, utilizing eco-friendly synthesized silver nanoparticles (AgNPs) through the LSPR (Localized Surface Plasmon Resonance) colorimetric method. Through this experiment, it was found that the recovery percentage averaged around 95%, indicating the reliability of this assay. Additionally, the Relative Standard Deviation (RSD) was consistently very nominal, highlighting the method's precision and consistency.

The fundamental basis of this innovative approach lies in its ability to exploit the unique interaction between AgNPs and melamine. The three amine groups within the melamine molecule were discovered to play a pivotal role, driving the aggregation of AgNPs, and forming the core principle behind this detection methodology. This simple yet effective process resulted in a substantial colour change in the presence of melamine, making it one of the easiest methods for detecting melamine in milk.

One of the outstanding features of this method is its inherent simplicity and user-friendliness. With an impressively low detection limit of 0.00475 gm/ml, it emerges as an exceptionally sensitive technique. Furthermore, the detection of melamine can be accomplished through a straightforward visual examination, eliminating the need for intricate instrumentation or convoluted sample preparation procedures. This user-centric attribute positions the assay as a promising solution for conducting on-site screenings to identify melamine adulterants in dairy products. Its expedited and uncomplicated operation, along with the successful optimization of the assay with minimal and simple changes, underscores its novelty and practicality. Moreover, the utilization of green synthesis further enhances its appeal as an eco-friendly and innovative method for melamine detection in milk samples, holding immense potential for ensuring food safety and quality assurance within the dairy industry.

In spite of its noteworthy advantages, it is essential to acknowledge certain limitations inherent to this detection approach. One notable drawback is its dependency on the human eye for visual assessment. While this attribute simplifies the analytical process, it also introduces subjectivity into the interpretation of results, potentially leading to variations in outcome based on the observer's perception.

Furthermore, the method's applicability may be restricted when dealing with highly complex sample matrices, which could introduce interference or false positives. Additionally, the detection limit of 0.00475gm/ml, while sensitive for many applications, may fall short of the requirements for exceptionally trace-level detection and this is the need of further study. Moreover, the stability and shelf-life of the silver nanoparticles in practical storage conditions may be a concern, as nanoparticles can be susceptible to aggregation or degradation over time. Lastly, the method may not be suitable for identifying certain melamine derivatives or analogues, as its specificity is tailored to the molecular structure of melamine itself.

While this method exhibits remarkable promise, particularly for rapid screening purposes, it is imperative to be cognizant of these limitations when considering its application in various context.

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