Investigation of Food Adulteration in Milk Products using Gold Nanoparticle based Surface Plasmon Resonance Probes

A dissertation submitted
in partial fulfillment of the requirements
for the award of degree of

Masters of Technology
In
Metallurgical and Materials Engineering

Submitted by

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DECLARATION

I hereby declare that the work being presented in this thesis entitled **"Investigation of Food Adulteration in Milk Products using Gold Nanoparticle based Surface Plasmon Resonance Probes"** by me in partial fulfillment of the requirements of the award of degree of **Master of Technology** in **Metallurgical and Materials Engineering** from **School of Physics and Materials Science**, Thapar University, Patiala is an authentic academic record of my experimental work carried out under the supervision of **Dr. B.N. Chudasama, Assistant Professor, School of Physics and Materials Science, Thapar University**. The matter presented in this thesis report has not been submitted in any other University for the award of Masters of Technology or any other degree.

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CERTIFICATE

This is to certify that the report entitled **"Investigation of Food Adulteration in Milk Products using Gold Nanoparticle based Surface Plasmon Resonance Probes"** submitted by Neetu Sharma 601302002 is a record of candidate's own work carried out under my supervision. To the best of my knowledge, the content of this thesis does not form a basis for the award of any other degree at Thapar University, Patiala or any other university.

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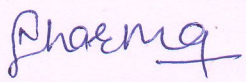
The explaination of my life and work is incomplete without paying regards to my respected and loving parents whose blessings and continuous encouragement have always shown me the path to achieve my goals. Above all, I express my indebtedness to the almighty for his love and blessings.



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Abstract

The main objective of this thesis is the detection of food adulteration using gold nanoparticle based surface plasmon resonance probes. This is a surface sensitive technique mainly used to detect analyte or target molecules using nanosubstrates. Here this technique is used to detect food adulteration and contamination in milk products using gold nanoparticles. “Melamine” is a chemical compound generally present in milk based food products mainly in liquid milk, infant formula powder and pet food. Although melamine is not allowed to use as an additive in milk based products however it is illegally added in pet food and infant formula powder to increase apparent crude protein content based on total nitrogen as it contains 66% nitrogen by mass. The excessive intake of melamine can result in the formation of insoluble melamine cyanurate crystal in kidney and can cause renal failure which may lead to uncertain death of pets and infants. In this dissertation a method is developed to analyze milk adulteration in infant formula powder by visual inspection. The method works on the principle that in the presence of melamine GNPs gets aggregated and show a visual color change from ruby red (due to spherical GNPs) to blue (due to aggregation of GNPs). In the presence of melamine, plasmon peak of GNPs shifts from 524 nm to 650 nm. The reason for the aggregation of GNPs are due to the amino group and ring nitrogen of melamine which strongly bind to the surface of citrate stabilized GNPs by the ligand-exchange and this ligand-exchange decreases the electrostatic repulsion between individual GNPs and finally results in the aggregation of GNPs. GNPs based surface plasmon resonance probes can be used to detect less concentration of melamine (2.41 mM) in infant formula powder. This detection limit of melamine is high as compared to Food and Drug Administration (0.01 mM) in infant formula powder but the method can be still used due to its simplicity, rapidity, low cost and reliability in both qualitative and quantitative capabilities.

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List of Abbrevations

SPR……...Surface Plasmon Resonance

LSPR……Localized Surface Plasmon Resonance

GNPs……...Gold Nanoparticles

SERS……...Surface Enhanced Raman Scattering

FDA……Food & Drug Administration

HPLC……High Performance Liquid Chromatography

HPLC-MS…….High Performance Liquid Chromatography-Mass Spectroscopy

LC……Liquid Chromatography

PEO- PPO-PEO…….Poly (ethylene oxide)-Poly (propylene oxide)-Poly (ethylene oxide)

CTAB……..Cetyl Trimethylammonium Bromide

ppm……..parts per million

ppb……parts per billion

ppt…….parts per trillion

LSPR-LS……...Localized Surface Plasmon Resonance-Light Scattering

PolyTn……...Polythymine

DBA…….Dihydroxybenzoic Acid

MA…….Mercaptopriopionic Acid

DTNBA……....Dithiobis Nitrobenzoic Acid

LOD…….....Limit of Detection

LOQ……..Limit of Quantification

DI……...Deionized

MPY……..Mercaptopyridine

EDS…….**....**Energy Dispersive Spectroscopy

JCPDS…….Joint Committee on Powder Diffraction Standards

FDA…….....Food & Drug Administration

Chapter 1 Introduction

1.1 Gold Nanostructure

Nanoscience and nanotechnology are recently discovered new approaches of science and engineering that are extending at a very fast speed. The main reason of their extending is the demand of fabricating newer materials with better properties and improved applications which affects near about all areas of science and engineering. In nanoscience and nanotechnology, we are talking about nanoparticles which have their size range between 1-100 nm in diameter whether they are suspended in any kind of medium i. e. solid, liquid or gas. Nanoparticles are a number of atoms or molecules held together. Their sizes are intermediate between single atom and bulk solid materials. Among all categories of nanoparticles, metal nanoparticles are the one type of particles which have special chemical, optical, magnetic and electronic properties that are significantly different from those of a single atom as well as their bulk phase material [1]. Optical properties of metal nanoparticles possess artistic, scientific and intellectual importance.

Now days colloids of metallic nanoparticles mainly gold, silver and copper have attracted much attention because they show characteristic colors in their spectrum. A number of colors obtained from gold solutions have provided a large study of their optical properties in an effort to correlate their nature under various environmental conditions. The prominent attributes displayed by gold colloids have been used in colored glasses from a long period. The first research work on gold sol was addressed by Michael Faraday. The formation of deep red solution of gold colloids by reducing hydrogen tetrachloroaurate solution was demonstrated by him. Gold samples can be prepared with particles that are smaller than light wavelength and because of this change in their particle size they gave rise to different colors in gold solutions. Faraday proved that it was the correlation of light with particles of different shapes and sizes that gave rise to the colors and the fact takes place due to the intrinsic colors of gold [2].

Due to their distinctive properties and potential applications gold nanoparticles have attract much interest (Figure 1.1). There are a wide range of studies are available on optical properties of individual gold nanoparticle in literature. Due to the reduction in reflectivity of light at the end

of the spectrum bulk gold sols posses a familiar yellow color [3]. The ratio of radius to the wavelength play a main role when gold is divided into finer to finer particles and the same happening is also occurs when the particles are smaller than the wavelength of light. When plasmon excitation is present in gold nanoparticles i. e. when the radius of nanoparticles is large in comparison to the wavelength of light the Rayleigh approximation (i.e. no retardation) holds and in that case the retardation effect should be included [4]. When the particle size of gold sols are very fine their color is ruby red which resulted from their strong absorption of green light corresponding to the frequency at 520 nm at which plasmon resonance occurs in gold. There are many other metals which also show this kind of resonance condition but in general the resonance frequency lies in the visible region near the ultraviolet. Their application mainly depends on this accidental combination of properties which showed them in a diverse range of niche applications. The intrinsic properties of metal nanoparticles mainly depend on their shape, structure, composition, size and crystallinity. These parameters could be easily control to fine-tune the properties of these nanoparticles. The color of these nanoparticles can be systematically varied from pink through violet to blue when such tiny particles are allowed to coalesce in a controlled manner [5].

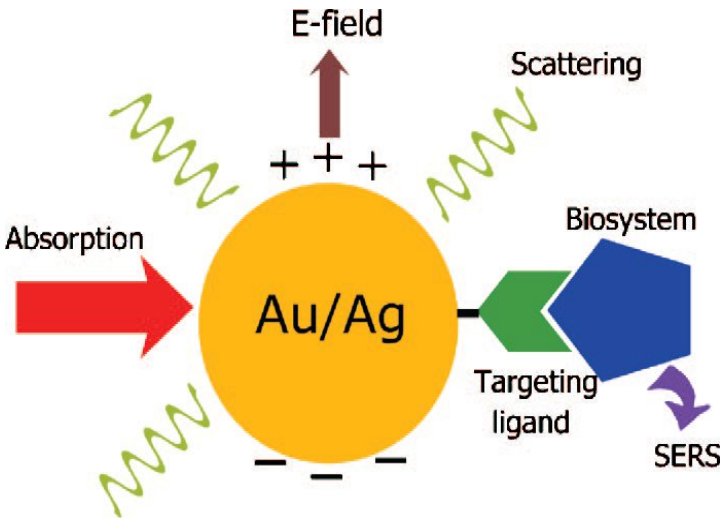

Fig.1.1. Nobel metal nanoparticle showing strong scattering and absorption of light [1].

1.2 Surface Plasmon Resonance

This is a phenomenon which mainly caused by the resonant oscillation of conduction electrons due to optical excitation. The phenomena persisted only at a certain angle of light and only at the thin metal-dielectric interface. The excitation process occurs when the frequency of incident light matches with the natural frequency of surface electrons which oscillates against the restoring force of nuclei (Figure 1.2) [6]. SPR is the basic concept for many instruments which are used for measuring adsorption of material onto the surface of metal nanoparticles or onto planar metal surfaces [7]. The surface plasmon resonance waves originate near surfaces which propagate in a parallel direction to the dielectric interface. The oscillations are very sensitive to any change of the boundary because the oscillating waves existed on the boundary of the metal and the external medium i.e. the adsorption of molecules on the metal surface.

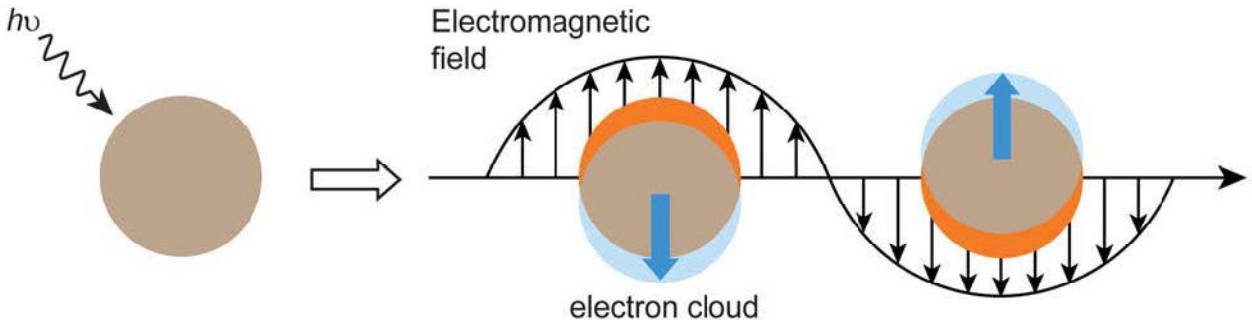

Fig.1.2. Collective oscillation of conduction electrons in response to optical excitation [6].

Localized surface plasmon resonance (LSPR) existed due to the collective oscillation of electrons in metallic nanoparticles when light is imposed on them. Enhanced near-field amplitude is shown by them at the resonance wavelength. The field shows high localization at the nanoparticle and decays at a very fast rate away from the nanoparticle interface into the dielectric background because of the far-field scattering by the particle. LSPRs provide the light intensity enhancement and localization which shows that the LSPR has a very high spatial resolution which is limited by the size of nanoparticles. Magneto-optical effect is also going to enhance due to the enhanced field amplitude effects that depend on the amplitude [8].

The surface sensitivity of several spectroscopic measurements including fluorescence, Raman scattering and second harmonic generation is going to improve by surface plasmons. SPR reflectivity mensuration in their simplest form can be used to detect molecular adsorption i. e. polymers, DNA, proteins etc [9]. The observations are depend on adsorbing molecules which cause changes in refractive index and bring the change in resonance states of the surface plasmon waves. The localized surface plasmon oscillations can give rise to different colors of suspensions or sols containing nanoparticles. Noble metal nanoparticles or nanowires exhibit strong absorption band in the ultraviolet-visible light regime that are not present in the bulk phase materials.

1.3 Surface plasmon resonance in Gold Metal Nanostructures

Due to their distinctive properties including enhancement in the optical field which results in the strong scattering and absorption of light, gold metal nanostructures have attracted much interest [10]. The main reason for improving the optical and photo thermal properties of metallic gold nanoparticles is due to the resonant vibration of their free electrons in the presence of light known as localized surface plasmon resonance. Figure 1.3 shows the origin of this phenomenon in gold metal nanostructures. This plasmon resonance process can either scattered light or be rapidly transformed it into heat (absorption). The latter process has find applications in several new fields.

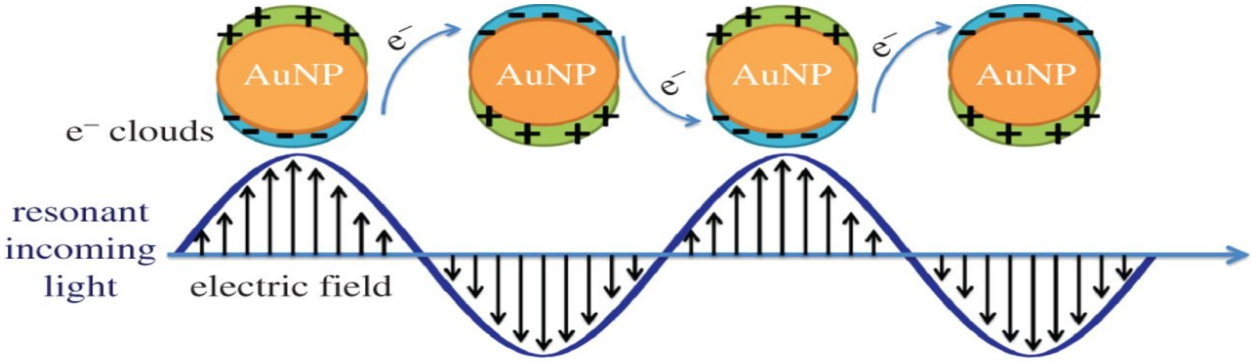

Fig.1.3. Origin of surface plasmon resonance in GNPs due to interaction between electrons in the conduction band of GNPs with incoming light [10].

The combination of gold metal nanoparticles with biological systems has put its great effect on biomedicine. Because of the strong LSPR scattering of gold nanoparticles, which when combined with specific targeting molecules, admits the molecular specific imaging and diagnosis of diseases such as cancer [11]. By varying the particle size, shape, composition and medium the gold nanoparticles can be finely tuned allows chemists to design nanostructures geared for specific biomedical applications [12]. For nanoshell or nanorod structures, the LSPR can be tuned to near infrared region making it easy to perform *in-vivo* imaging and therapy.

1.4 Food Adulteration

"Adulteration" is a juridical term means that a food product is unable to meet state standards. Adulteration mainly used to refer dissentience with health or safety standards as defined in the United States by the Food and Drug Administration and the U.S. Department of Agriculture. The Federal Food, Drug and Cosmetic explained that food gets "adulterated" if it gets contaminated from any one of the following:

Poisonous Substances

If any food contains a poisonous material that may proves it harmful for health, it is adulterated. But, if the poisonous substance is intrinsic or occurred naturally and if its quantity in the food does not render it injurious to health, the food will not be considered as adulterated. Thus if a food contains any natural infection at very low levels that would not ordinarily be injurious is not adulterated [13]. And if the poisonous or deleterious substance is unavoidable and is within safety limit or action level the food will not be considered to be adulterated.

Foreign Matter

These material include any objectionable substances in foods such as remote matter (i. e. metal, glass, wood, stones, plastic, sand), undesirable parts of the basic plant material (i.e. pits in pitted olives, stems, pieces of shell in canned oysters) [14] and sewage waste (mainly rot, insect, mold, and rodent parts, decomposition and excreta). According to strict reading of FD&C Act, any amount of waste and unwanted matter in food would render it adulterated.

Economic Adulteration

A food is adulterated if it exclude a valuable component or replace another valuable component completely or in some parts i.e. for a valuable constituent (e. g. tree oil is used in dilution of olive oil), conceals damage or lowliness in any manner (e. g. food colorings are used on surface of fresh fruits) and any unwanted substance has been put in it or packed with it to enhance its bulk weight, degrade its quality or strength and just to appear it of greater value than it is [15].

Milk Adulteration with Melamine

Milk adulteration with melamine of milk-based food stuffs is now a great issue. Melamine is not permitted to use as an ingredient in food stuffs. It is now well know that this was due to the combination of melamine and cyanuric acid found in pet food. While last few year records showed that melamine was unfairly added in pet and human food to increase apparent crude protein value as it contains 66% nitrogen by mass in some developing countries [16]. But the uncontrolled amount of melamine will result in the evolution of insoluble melamine cyanurate crystal in kidney and cause to renal complication.

Food and Drug Administration of USA and European Union have setup a protection limit of melamine 2.5 mg kg^{-1} in milk and milk stuffs. The maximum remainder level of melamine in infant formula and other dairy products are lawfully adjusted at 1 and 2.5 mg kg^{-1} respectively. Therefore there is a good need for establishing a rapid and reliable analytical method by which the detection of melamine in milk-based products will be possible at level desired by regulatory authorities.

1.5 Detection of Food Adulteration Using SERS

By using SERS technique with metallic nanostructures has overcome the problem of weak signals with the conventional Raman technique. Most of the analytes (contaminants and food additives) are present in very less concentration in food samples. Therefore, SERS is an auspicious instrument for fast and sensitive detection of these chemicals [17].

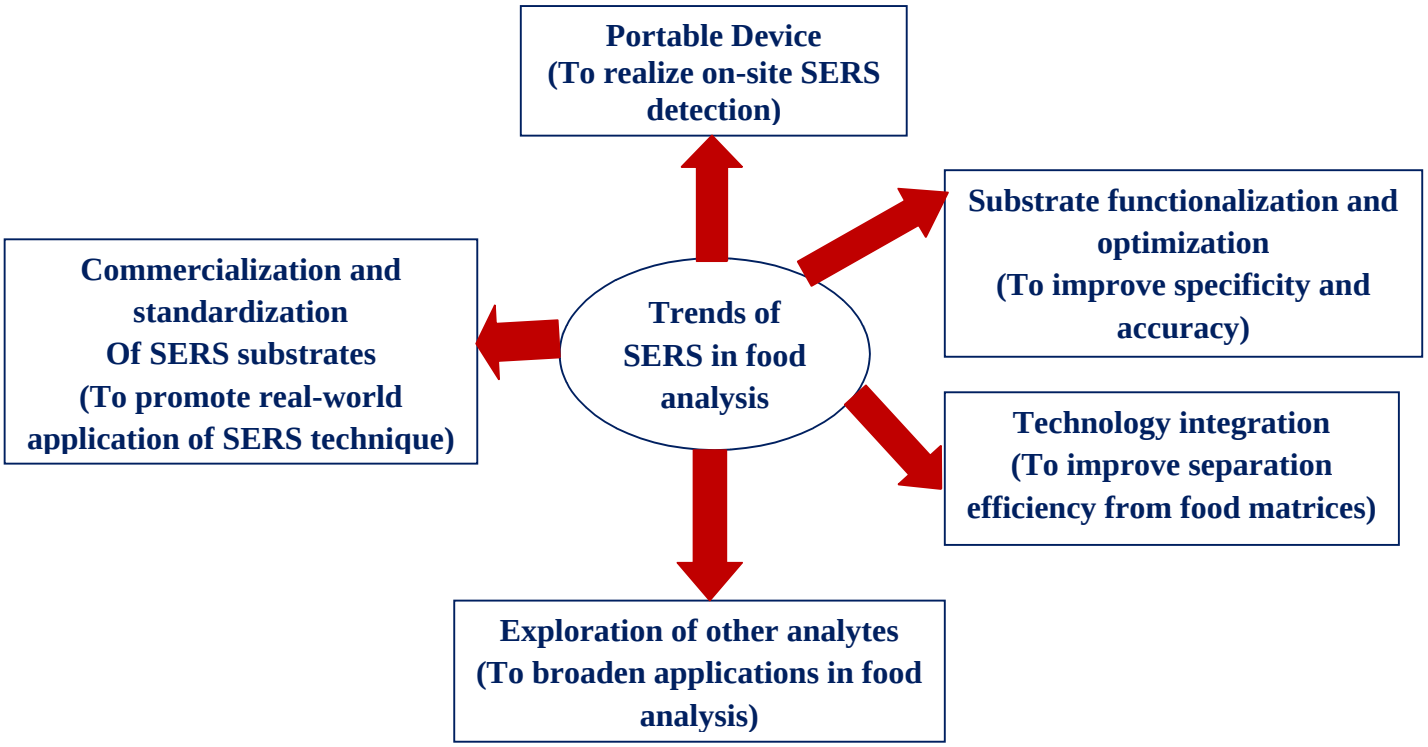

Fig.1.4. Trends of Surface Enhanced Raman scattering technique in food analysis [17].

Recent SERS analysis on chemical inspection of food is still in the early stage, as most of the studies have concentrated on substrate development and assessment using these analytes in simple solvents. Although SERS posses several benefits in food adulteration but one major problem arise is due to its high cost availability. By using cheap portable Raman instrument for SERS detection the cost for application and for on-site detection can be reduced. To advance the SERS technique in real applications of food analysis further research work is required as shown in Figure 1.4 (1).SERS substrates commercialization and standardization, (2).Integration of technology, (3).Substrate optimization and functionalization, (4).Use of a portable device for on-site SERS detection and (5).Explorating other applications in food analysis. Recently there are a great need for developing rapid, sensitive and reliable methods to detect food hazards as is seen by food safety incidents and public health concerns about synthetic food additives and chemical residues. SERS is used as a quantitative or qualitative detection technique for modern chemical analysis of food. We can classify the analytes into food additives and chemical contaminants from a compositional perspective.

Food additives

Food additives or food supplements are the substances that become part of a food product when added during handling or making of that food. Food additives mainly include colorants, preservatives, texture modifiers, flavors and flavor enhancers, nutrients and others. There has been a great increase in the use of food additives with the increase in use of canned foods over the last few decades. Public health concern over artificial food supplements are also increasing continuously. The SERS studies of last few years have been applied on food supplements while most of them were still at the early stage of characterization. The target analytes which have been investigated till now include antioxidants like butylated hydroxyanisole, antimicrobial agents such as benzoic acid derivatives, the flavor enhancer monosodium glutamate, sweeteners, nutrients like flavones and iodide ion.

Most of these studies were mainly concentrated on the adsorption nature of food additives on the exterior of metal nanoparticles by differentiating the attributes of enhanced and normal Raman spectra. Occurrence of new bands as well as shifts in the observed SERS estimated with Raman signals provides structural information useful for the clarification of interaction between the metal and the adsorbate.

Chemical contaminants

Chemical contaminants in food mainly consist of chemical adulterants, agricultural and environmental, mycotoxins and foreign food ingredients. Mainly antibiotics, pesticides, illegal food colorants, illegal drugs, foreign allergenic and toxic proteins and melamine are the most common targets in foods that have been studied using SERS. SERS can also be used to study environmental pollutants i. e. toxic metals like mercury, copper, chromium, cobalt, lead, arsenic and others. But their major target is generally drinking water not food products.

Melamine

In last few years milk contamination with melamine in food caused serious public health issues and enormous economic losses. In these happenings melamine was illegally added in food to

elevate the estimated protein content. After this event FDA has set up a safety limit for melamine i.e. 1 ppm and 2.5 ppm for infant formula and milk based food products respectively. Consequently, it becomes essential for industries and government agencies to adjust the value of residual melamine. Many techniques have been investigated and applied in sensitive, rapid and accurate detection of melamine i.e. HPLC-MS, LC, and HPLC and so on [18]. Existing scientific methods that are used to measure melamine and related ingredients in foods for human consumption and animal feeds were presented by Tittlemier.

Due to a good sensitivity of SERS, it can be used as a best tool which provides a great benefit for fast detection of melamine. A large number of SERS-active substrates are developed in the past several years to detect melamine. Melamine can be quantitatively and qualitatively determined using SERS as the Raman peak of melamine generally in the range of 670 to 710 cm^{-1} approximately. So to observe the limit of detection of different nanosubstrates SERS is a best technique using melamine because it posses unique sharp peak and prominent safety concern. Various SERS substrates have been invented due to their different sensitivity and quantification ability for the analysis of melamine. Efforts have also put to detect melamine with little sample preparation or no sample preparation. Most substrates used for melamine detection exhibit great sensitivity range near about 10^{-7} to 10^{-8} M for melamine detection for food products.

Chapter 2 Literature Review

Various studies on synthesis of spherical gold nanoparticles and food adulteration were carried out by researchers in the past. This chapter reviews the literature which lays foundation and basis for thesis work. This helps us to give a better understanding about the topic and also acts as a guideline for our thesis. In this chapter, theoretical and experimental approaches on synthesis, characterization and detailed theories for describing the experimental results have been explained**.**

Firstly in 1951 Turkevitch gave a method of synthesizing spherical gold nanoparticles by reducing $HAuCl_4$ by citrate in presence of water and is now commonly referred as ‘‘Turkevitch method’’. Since then this method has been improved by a number of researchers to produce spherical gold nanoparticles having wide range of diameters from 15 to 150 nm which can be done by employing γ-radiation method or by controlling the ratio of citrate to $HAuCl_4$.

There are a variety of methods which have been demonstrated in literature to achieve a better control on the size and uniformity in size, one of the best approach is ‘‘Schmidt method’’ which reported in 1981 and the ‘‘Burst–Schiffrin method’’ presented in 1994. Another technique which is commonly used for producing spherical gold nanoparticles of different sizes is “Seed-mediated growth”. The size of gold nanospheres could be tuned from 5 to 40 nm by taking a better control on the ratio between precursor and seed which has been demonstrated by Murphy and coworkers.

The formation of gold nanospheres can further reviewed by Daniel and Astruc. The color which is shown by gold nanospheres i. e. ruby red can be described quantitatively by assuming the scattering of light of an electromagnetic wave from the nanoparticles. Some recent literature related to synthesis of spherical gold nanoparticles, up comings and drawbacks of each synthesis is summarized in table 2.1.

2.1 Synthesis of Spherical Gold Nanoparticles

Table 2.1. Literature on synthesis of GNP's

Year	Writer	Research
2004	**Toshio Sakai & Paschalis Alexandridis**	Single-step synthesis of GNPs using $HAuCl_4$ and PEO- PPO-PEO block copolymer [19] **Advantage:** The synthesis procedure is environmentally benign and economic. The method proceeds fast to completion and results in a "ready-to-use" product. **Disadvantage:** GNPs having average dia of 10 nm can be synthesized only i. e. by varying the ratio between gold and reducing agent particle size cannot be varied.
2005	**Hussain *et al*.**	Single-step synthesis of GNPs using $HAuCl_4$ and thiol capping ligand [20] **Advantage:** Particle size control can be achieved precisely by taking ratio of gold to capping ligand and in both kind of solutions i. e. aqueous and non aqueous, it can be easily obtained. **Disadvantage:** Nearly monodisperse gold nanospheres in the size range of 1-4 nm can be synthesized only.
2006	**Kimling *et al.***	Synthesize GNPs by reducing citrate and ascorbic acid [21] **Advantage:** GNPs formation can be possible in a wide range i. e. from 9 to 120 nm which is within defined size of distribution. **Disadvantage:** To initiate the reaction thermal activation is required. Cluster formation and collapse to larger particles may take place during synthesis.

2009	**Xia *et al.***	Synthesize GNPs by $HAuCl_4$, sodium citrate and silver nitrate [22] **Advantage: By using this** method the effect of citrate to buffer the pH of the reaction can be easily controlled and thus change the type and reactivity of auric ions and increase the nucleation and growth rate of gold nanoparticles. **Disadvantage:** The sizes of quasi-spherical gold nanoparticles obtained increases from 12 nm to 36 nm.
2009	**Jana *et al.***	Synthesize GNPs by wet chemical method [23] **Advantage:** The observed nucleation can be exceedingly enhanced (99%) with compare to growth of particle. **Disadvantage:** By adding the reducing agent slowly the seeds do grow but in the produced gold nanoparticles possess a less homogeneity in shape.
2009	**Azam *et al.***	Synthesize GNPs by microwave irradiation approach using citric acid and cetyl trimethyl ammonium bromide (CTAB) as reducing agent [24]. **Advantage:** This method was very rapid and the assembled product was very stable. **Disadvantage:** The expedition reaction is completed under microwave irradiation in short duration and can be applied to generation of spherical gold nanoparticles.
2011	**Bastus *et al.***	Synthesized GNPs having narrow size distribution and uniform quasi-spherical shape up to 200 nm by kinetically controlled seeded growth strategy [25]. **Advantage:** Gold nanoparticles can be further functionalized with a large number of molecules.

		Disadvantage: Secondary nucleation during homogeneous growth
2011	**Roshdi Seoudi And Doaa A. Said**	Synthesized GNPs by different capping materials i.e. sodium citrate, cetyl trimethylammonium bromide (CTAB) and chitosan [26]. **Advantage:** Particle size can be easily controlled by the variation of molar ratio between sodium citrate, CTAB and chitosan to Au^{3+}. **Disadvantage:** Aggregation of particles
2014	**Evelina Polievkova**	Synthesized GNPs by green method [27] **Advantage:** Used for various biomedical applications including *in-vivo* and *in-vitro* bioimaging techniques. **Disadvantage:** Cytotoxicity of as-synthesized GNPs

2.2 Detection of Milk Adulteration

Guo *et al.* [28] demonstrated an approach to detect melamine in the presence of GNPs by melamine induced color change of GNPs from wine-red to purple due to the aggregation of GNPs, which works as a basic concept for rapid and field-portable colorimetric detection of melamine. They showed that the approach can be used to detect melamine in liquid milk and infant formula which provide a detection limit from 1.0 ppm and 4.2 ppm by investigating with naked eyes without using any advance instrument and without need of any more treatment of sample and it can be used to detect very less concentration i.e. 0.15 ppm of melamine in liquid milk and 2.5 ppm of melamine in infant formula with the use of UV–Vis-spectroscopy. It was limited due to its large duration of measurement (30 minutes) by naked eye observation.

Li *et al.* [29] reported the simple technique to detecting melamine in raw milk using GNPs as a probe. The technique is based on GNPs-distance dependent optical properties. It was shown that

the aggregation of GNPs takes place very fast in neutral media and thus results in color change from red-to-blue. The quantification of the concentration of melamine in raw milk can be possible with UV–Vis spectroscopy and by naked eye examination. The recent limit of detection for melamine is 0.4 ppm. The advantages i. e. simplicity, rapidity, low cost and visual colorimetry make the method very useful for on-time testing of melamine within the safety limit in raw milk.

Chi *et al.* [30] reported a sensitive, reliable and simple approach for visualization of melamine in milk products using citrate-stabilized GNPs. After interaction with ppb-level melamine, GNPs solution shows a clear color change from red to blue and fast rate of aggregation within 5 minutes, it can be seen directly with the naked eye and can also be by using UV-Visible absorbance spectra. After comprising with other typical amino group compounds it can be emphasized that, the melamine is a compound which contains a large number of binding sites at the GNPs-surface and thus play an important role as a linker molecule to provide efficient crosslinking of GNPs. But to improve the sensitivity $NaHSO_4$ is used which cause the ligand exchange process between citrate at GNPs surface and melamine.

Ai *et al.* [31] developed a nanoparticle sensor for visual melamine detection. It was showed that the technique does not acquire any complex instruments, by which operations are easy to handle and cost effective. The method shows 2.5 ppb detection limit which is very low for naked eye observation within 1 minute, which can be used for ultrasensitive detection of melamine in milk related products. The sensor posses excellent selectivity for melamine with respect to other molecules having same structures and also for the residual ingredient in the extract obtained from raw milk and infant formula. The method provides a promising root for real-time melamine detection of raw milk, infant formula and other products of milk.

Wei *et al*. [32] developed a process for facile detection of melamine in products made by milk and related ingredients. In the process specific precipitation obtained by GNPs and cyanuric acid are added to study colorimetric signal readouts, which results to exact melamine detection. The main advantage of the method was good sensitivity measurement i. e. 40 ppb with a two standard deviation cutoff. But when pH level was greater than 6, at that time the separation efficiency was

low and the interference from remaining milk components could not be removed, which resulted in poor sensitivity.

Qi *et al.* [33] detected melamine by interacting it with polythymine (polyTn) which formed polythymine (polyTn) modified GNPs in aqueous medium due to the formation of triple H-bonds, which resulted in aggregation of the (polyTn) GNPs. Due to the variation of localized plasmon resonance, the color of the mixture solution is going to change from wine red to purple and localized surface plasmon resonance light scattering (LSPR-LS) signals are going to enhanced.

Cao *et al.* [34] developed nonaggregation-based and label-free colorimetric probes of GNPs for melamine detection. 3, 5-dihydroxybenzoic acid (DBA) is used as a reducer for generating GNPs without addition of gold nanoparticle seeds at room temperature. After melamine addition, DBA do react with melamine by interaction of strong hydrogen-bonds. Thus, the GNPs formation was obstructed by melamine because no more reduction of Au^{3+} ions was possible. Due to insufficient amount of reducer the color was going to change from purple to yellowgreen with increasing melamine concentration. The plasmon absorbance of GNPs allows the melamine detection quantitatively. A linearity existed between the logarithm of melamine concentration ranging from 1×10^{-9} M to 1×10^{-5} M with absorbance having a linear co efficiency of 0.993.

Cai *et al.* [35] demonstrated a new approach for melamine detection using 3-mercaptopriopionic acid (MA) and GNPs. The process is worked on hydrogen-bonding recognition and electrostatic interaction between MA and melamine. GNP surfaces are conjugated by MA molecules to form MA-modified-GNPs, which act as nanoprobes in melamine detection. Aggregated nanoparticle mediated signal amplification can be examined by absorption spectroscopy, the method provide detection limit of 0.4 μg/mL as a sensitive technique, and the linear detection is ranged from 0.6 μg/mL to 42 μg/mL. The color change can be observed by naked eye at 30 μg/mL melamine without the requirement of any advanced instruments.

Yazgan *et al.* [36] reported a technique for sensitive melamine detection based on SERS. GNPs having shape of spherical magnetic-core shell and rod-shaped labeled by a Raman-active

compound was used as melamine molecules to form a complex. Due to easy adsorption of 5, 5′ dithiobis (2-nitrobenzoic acid) (DTNB) it is readily used as Raman active compound which gets adsorbed by GNP surface forming a self-assembled monolayer and posses strong Raman scattering at 1330 cm^{-1}, due to the symmetric NO_2 stretch. The calibration curve was obtained by taking melamine concentration versus Raman band area at 1330 cm^{-1}. A good linear relation was observed with a high determination coefficient. LOD and LOQ were obtained as 0.39 mg/L and 1.30 mg/L for samples spiked with melamine.

Mecker *et al.* [37] described a technique for detecting melamine in a number of solid matrices at the 100–200 μg/L level using SERS and GNPs. With the help of portable Raman spectrometer and easy sample preparation, the work shows field-based screening of adulterated melamine. For both type of analysis i. e. colorimetric and Raman-based, citrate coated GNPs were investigated. For developing a melamine extraction procedure several non-hazardous solvents were evaluated which have a good safety for field applications. Raman signals of melamine and eliminated matrix can be enhanced by GNP agglomerates which are formed due to the addition of isopropanol prior to sample introduction. The enhancement resulted due to melamine Raman signal is 10^5 by using GNP agglomerates.

Kim *et al.* [38] developed a concept for detecting melamine concentration using portable sensor using gold nanofinger structures and SERS. Due to the use of authentic gold nanofinger SERS chips having high performance, the LOD of melamine in DI water was estimated to be 120 ppt without any pretreating of sample. A simple aspect of detecting very less amount of melamine below the FDA limit can be achieved using this system, for sensing of melamine in milk products. The sampling processes used are either dialysis or gel-filtration which are one-step and easy to use processes and within a limited-resource environment fully compatible for field applications. The limit of detection of melamine identified by gel filteration method is 100 ppb in whole milk and infant formula, which is lower than 1 ppm (FDA limit of infant formula). But the high cost and expensive instrumentation limits the method.

Lou *et al.* [39] developed SERS based strategy by 4-mercaptopyridine (MPY)-modified GNPs for sensitive and exact identification of melamine in milk powder. The measurement for SERS

strongly relied on the "hotspot" effect, in which melamine induce aggregation within GNPs immediately, which enhance the Raman intensity of the reporter molecule MPY and provide a significant color change of sample solution from red to blue-gray. The investigated limit of detection was found to be 0.1 ppb of melamine, with a linearity of 0.5–100 ppb, which proves that a wider quantization range and good sensivity can be achieved than direct SERS sensing methods. The method possesses good applicability for in situ examination of melamine in complex matrices.

He *et al.* [40] measured ocillational spectroscopic characteristics of melamine, melamine cyanurate and cyanuric acid using gold nanosubstrates coupled with SERS. Small concentration of cyanuric acid, melamine and melamine cyanurate was quantified and characterized quickly and exactly by combining SERS with partial least squares analysis. By analyzing the log values of concentration of melamine and the relationship between the Raman intensity of the most prominent peak at around 676 cm^{-1}, the LOD of SERS for melamine was found to be 33 ppb. When melamine and cyanuric acid were added in equal amounts, due to the formation of melamine cyanurate the spoke like crystals formed instantly, which could be characterized and measured by SERS. But for cyanuric acid the acquired SERS spectra are very differ from this compound in an aqueous solution, showing a reaction of cyanuric acid in water. But for the whole process sample pretreatment is essential.

Giovannozzi *et al.* [41] presented a rapid detection technique to detect melamine in liquid milk, based on SERS exploiting the selective binding of GNPs with analyte. It was found that the interaction promotes the aggregation of the GNPs inducing a huge enhancement of the melamine signals in the Raman spectrum due to the formation of SERS hot spots. A linearity ($R^2 = 0.99$) was estimated in the concentration range of 0.31–5.0 mg/L in milk with a detection limit of 0.17 mg/L. It was cleared that for the whole process a long extraction procedure is not required (total time for analysis can be less than 30 minutes) and it can be used reliably for melamine detection in complex milk matrix as according to European standards.

Table 2.2. Summary of other food adulterants

Analytes	Substrates	LOD	References
Food additives:			
Benzoic acid	Au colloids	-	Gao et al. [42]
Phthalic acid	Au colloids	-	Gao et al. [42]
Sodium benzoate	Ag colloids	-	Peica et al. [43]
Butylated hydroxyanisole	Au colloids	10 ppm	Yao et al. [44]
Aspartame	Ag films	-	Peica et al. [45]
Monosodium glutamate	Ag colloids	10^{-5} M	Peica et al. [46]
Iodide	Rh_6G-adsorbed Au colloids	30 ppt	Dasary et al. [47]
4-Arsanilic acid	Ag/polydimethylsiloxane	-	Fullerton et al. [48]
Roxarsone	Ag/polydimethylsiloxane	-	Fullerton et al. [48]
Acetarsone	Ag/polydimethylsiloxane	-	Fullerton et al. [48]
HMB	Ag colloids	-	Podstawka et al. [49]
L-Carnitine	Ag colloids	-	Podstawka et al. [49]
Creatine	Ag colloids	-	Podstawka et al. [49]
Illegal food dyes:			
Sudan I	Au colloids	48 ng/g	Cheung et al. [50]
	Electropolished Al	10^{-7} M	Di Anibal et. al [51]
Sudan II	ZnO/Ag nanoarrays	10^{-12} M	Hu et al. [52]
Sudan IV	ZnO/Ag nanoarrays	10^{-12} M	Hu et al. [52]
Ponceau 4R	Au colloids	5 ppm	Xie et al. [53]
Tartrazine	Ag colloids	10^{-10} M	Peica et al. [54]

Chapter 3 Experimental Techniques

This chapter outlines synthesis methods of spherical gold nanoparticles. Besides it, basic theories & principles for various experimental techniques like UV-Visible spectroscopy, Dynamics Light Scattering (DLS) and Transmission Electron Microscopy (TEM) which are utilized further for the characterization of GNPs are also described.

3.1 Synthesis of Spherical Gold nanoparticles

There are mainly two approaches by which spherical gold nanoparticles are prepared:

(a). Top-down synthesis
(b). Bottom-up synthesis

In top-down approach bulk metallic solids (in this case gold) is broken to prepare gold of required dimensions (Figure 3.1). Here size of nanoparticles and their structure are controlled by matrix or pattern. But the top–down methods [55] have many limitations in controlling particle size, shape and surface functionalization. The bottom-up methods of preparing gold nanoparticles by wet chemical method depends on the electrochemical process, chemical reduction of metallic salts and decomposition of organo-metallic compounds by controlling their sizes. In the bottom–up synthesis nanoparticles are made by their molecular components (Figure 3.1). This approach involves chemical or biological reduction containing two-steps nucleation and growth. When these two reactions are going to complete in the same process it is called in-situ synthesis otherwise it is called seed–growth. Firstly the building blocks are prepared by reducing the corresponding metal salts to zero valent state. The metal atoms tend to merge into larger units because they have a short life time in solution. Bottom-up approach is more popular as compared to Top-down approach because by using this approach particle size and shape can be easily controlled.

There are two important components in the preparation of GNPs accomplished by chemical reduction i.e. the chosen reducing agent and the stabilizing ligand. In liquid chemical methods,

GNP's can also be prepared in aqueous medium by reducing metallic salts with suitable reducers in presence of suitable stabilizing agent.

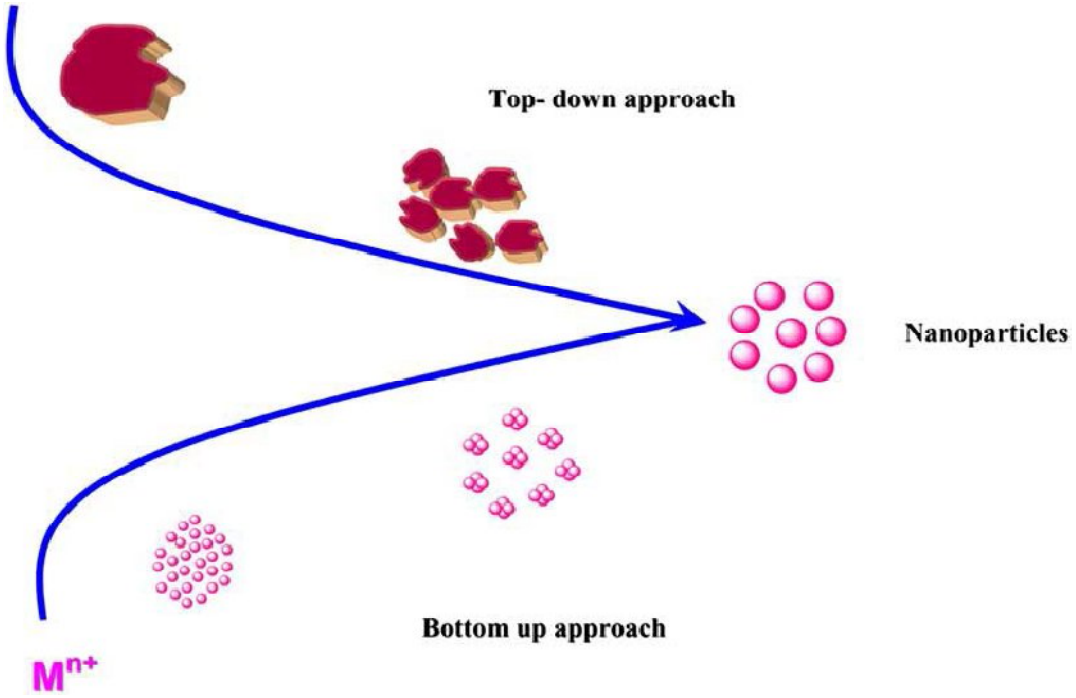

Fig.3.1. Top-down and bottom-up synthesis of gold nanoparticles approach [55].

Disadvantage of the nanoparticles synthesis by this approach is they have tendency to agglomerate, which is caused by high surface to volume ratio. The numbers of nanoparticles are adhered to each other in the presence of Vander Waal's force, so controlling an agglomeration is one of the major problem for GNPs synthesis [56]. Electrostatic stabilization and the steric stabilization are the two processes which are mainly used to achieve stabilization in nanoparticle matrixes. The electrostatic stabilization is defined as repulsive interaction between same charged nanoparticles (which stops them from aggregation). Gold nanoparticles prepared by citrate route are an example of this kind of stabilization. The main electrostatic stabilizers are alkane thiols and polymers. While steric stabilization is resulted due to steric repulsion between the giant hydrophobic moieties of the stabilizing agents.

3.1.1 Brust-Schiffrin method

This procedure is based on use of high yield two-phase water–toluene solution with a thiol as stabilizing ligand and tetraoctylammonium bromide as the phase transfer agent. Using phase-transfer reagent aqueous chloroaurate ions are transported into organic solvent and then these ions in the organic phase are reduced to zero valent Au and finally capped with alkane thiol or alkylamine molecules [57]. The main advantage the route posses is nanoparticles further functionalization by ligand exchange process.

3.1.2 Seed-Growth method

This synthesis method is worked on enlarging the particles step by step. The control over size and shape of as-synthesized GNPs at every step is very easy. This synthesis approach consists of two-steps. In the first one, small-sized seeds of GNPs are synthesized. Then, in the second one the prepared seeds are added to growth solution which contains stabilizing and reducing agents and $HAuCl_4$. After that these newly reduced Au° grow on the surface of seed to form larger sized GNPs. The reducing agents which are used during second step are mild. They provide reduction of Au^{III} to Au° only when Au seeds are present there as a catalysts, so the newly reduced Auº can gather on the surface of Au seeds [58]. The second-step having lower rate of reaction than the first-one because of the use of mild reducing agents. To continue the growth process this step can be repeated. The arrangement of the seeds affects the size, shape and surface properties and the ratio to the Au precursor during the seed-growth synthesis.

3.1.3 Turkevitch method of synthesizing GNPs

The most popular in-situ synthesis of GNPs is so called Turkevitch method published in 1951. This method is depend on the reduction process of solvated aurochloric acid by sodium citrate. In this method citrate can be used as stabilizing and reducing agent or as the stabilizing agent only.

Chemical Reagents

Hydrogen tetra chloroauret ($HAuCl_4$), trisodium citrate and chloroform were obtained from Sigma- Aldrich. Trichloroacetic Acid (AR Grade) and melamine (LR Grade) were obtained from S. D. Fine-Chem. India Ltd. Sodium carbonate (LR Grade) was obtained from Loba Chemie Pvt Ltd. Infant formula was procured from local supermarket. All solvents used are having analytical grade and used without any further purification Millipore Milli-Q (specific resistance of 18.2 MΩ/cm) water was used during whole experiment. All glasswares used in the experiment rinsed thoroughly in water and prior to use dried in air.

Synthesis of GNPs

To synthesize GNPs by this method 1 mM, 50 mL $HAuCl_4$ solution is boiled followed by quick addition of 38.8 mM, 5mL trisodium citrate dihydrate under dynamic stirring. The mixture was further refluxed for another 15 minutes. After cooling of the mixture to room temperature under continuous stirring the wine-red solution of GNPs was procured. The GNPs obtained are stored at 4 °C before use. The schematic representation of this process is shown in figure 3.2. The plasma resonance peak of GNPs was obtained at 524 nm and the hydrodynamic size of GNPs was ~ 56.2 nm.

This procedure has also formed the basis of consequential synthesis of different shapes of gold nanoparticles. Citrate is used as a stabilizing agent in the preparations of GNPs. The citrate adsorption on nanoparticles surface is very important in this procedure by which particle shape and size gots affected. In the first step, the particle size decreases meanwhile citrate to $HAuCl_4$ ratio increases this is called stabilizing process.

Higher amount of citrate allows the fast stabilization of small particles while lowers the concentration inflicts aggregation, which leads to larger form of particles because the stabilizing process is incomplete. While in Turkevich's method citrate is used both as a reducing agent and as a stabilizing agent, the evolution of this method now enables the utilization of a variety of reducing agents like sodium borohydride and a variety of stabilizing ligands.

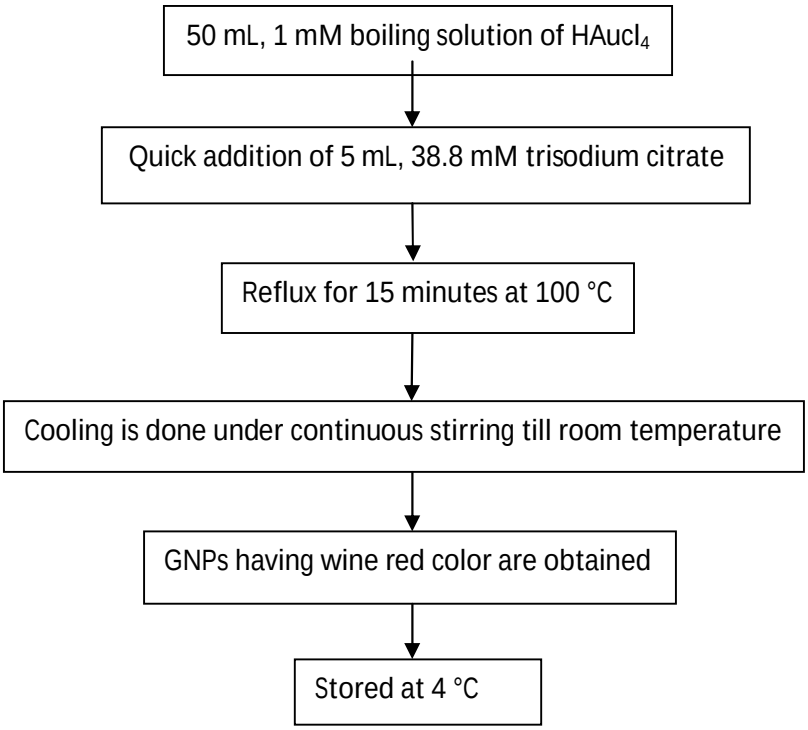

Fig.3.2. Schematic representation synthesis of GNPs.

3.2 Detection of Melamine

3.2.1 Detection of Melamine using GNPs

For detection of melamine, 2 mL stock solution (made by 0.126 g of melamine after dissolving in 1 L of water) was taken and added into 1 mL of GNPs solution. The mixture was then reacted for 3 minutes at room temperature. The absorption spectrum of the reacted solution was determined and the concentration of melamine was examined based on absorption ratio of GNPs at specific wavelength (λ_{max}) before and after the melamine addition. The schematic of this process is shown in figure 3.3.

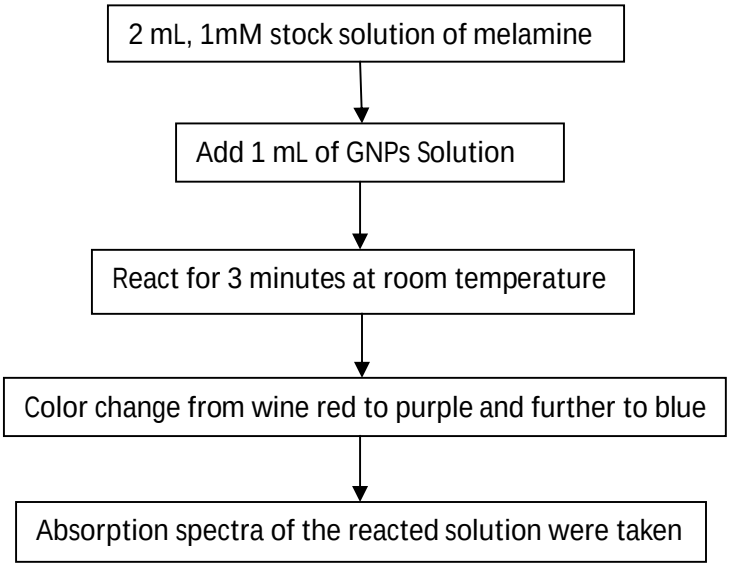

Fig.3.3. Schematic for the process of detection of melamine using GNPs.

3.2.2 Sample Preparation for Infant Formula Powder

For infant formula powder, about 0.6 gm of infant formula sample was used for the determination. After dissolving in 2.0 mL of water in a centrifuge tube, 2.0 mL of 10% trichloroacetic acid and 1.2 mL chloroform were added. The entire mixture was mixed and sonicated for 15 minutes using ultrasonicator. The mixture was centrifuged at 13,000 rpm for 10 minutes to separate the deposit.

The solution was then taken into another centrifuge tube and pH of the solution was adjusted to 7.0 with 1 M of Na_2CO_3. It was again centrifuged at 3000 rpm for 10 minute to separate the deposit and the solution obtained was used for melamine detection. The schematic of this process is shown in figure 3.4.

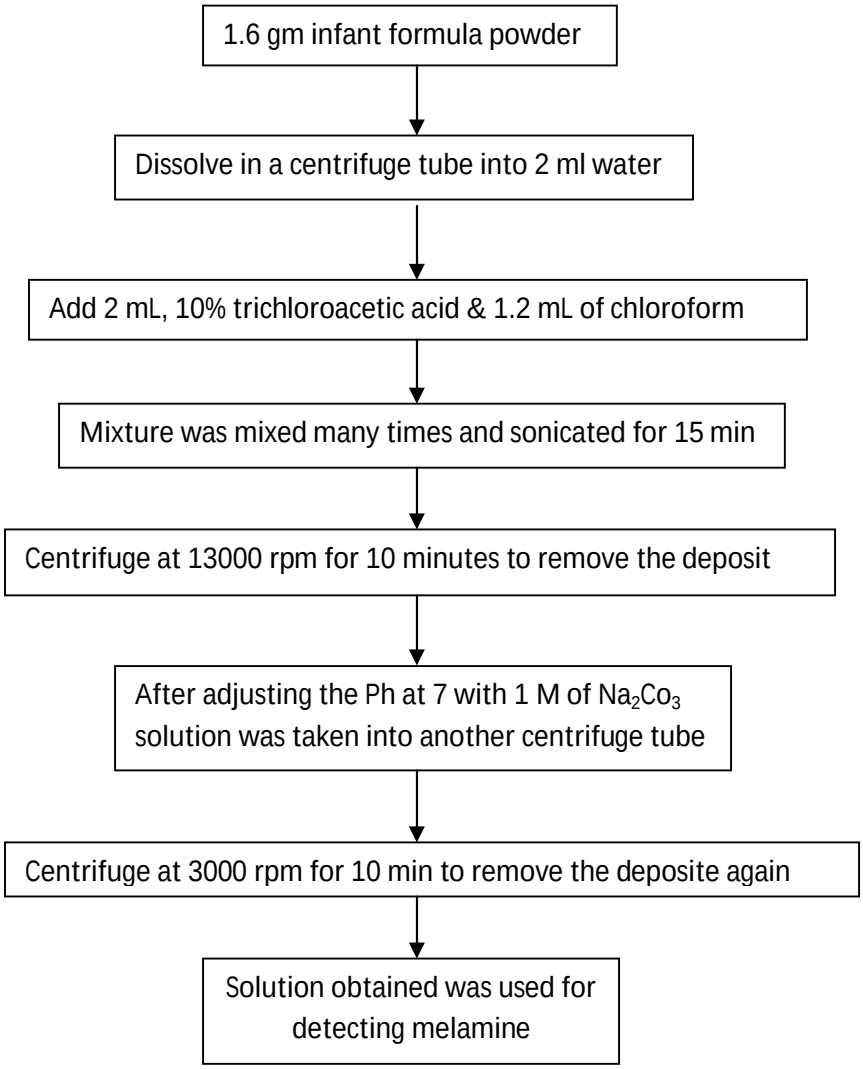

Fig.3.4. Schematic for the pretreatment of Infant Formula Powder.

3.2.3 Detection of Melamine in Infant Formula Powder

To detect melamine, 2 mL infant formula solution was taken with 1 mL GNPs solution. The mixture was reacted for 3 minutes at room temperature. The UV-Visible spectra of the reacted solution were observed and the concentration of the melamine was determined. The schematic of this process is shown in figure 3.5.

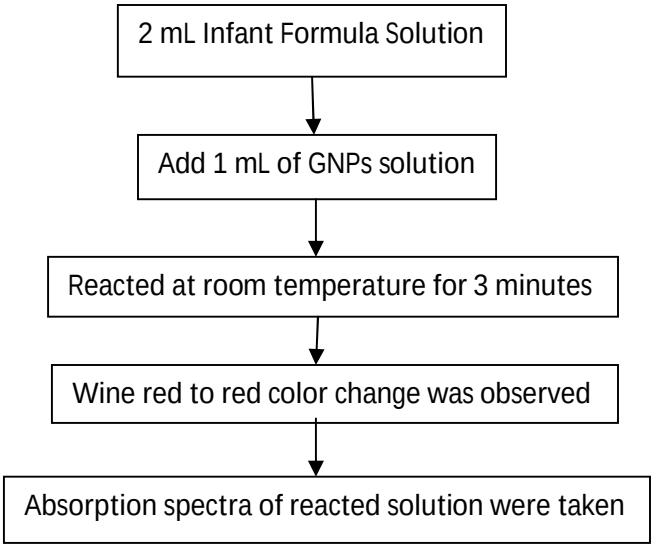

Fig.3.5. Schematic of the detection of melamine in Infant Formula Powder.

3.3 Characterization techniques

3.3.1 UV-Vis-spectroscopy

UV-Visible absorption spectroscopy is a spectroscopic technique in which light is used in visible, ultraviolet and near infrared ranges. UV-Visible spectroscopy is mainly used to refer absorption spectroscopy in near IR and ultraviolet-visible region. This is the region where molecules show their electronic transitions. Absorption of light occurs very quickly. The energy equation is given as:

$$\mathbf{E = h\nu = hc/\lambda}$$

Where h is the Planck's constant, E is the energy, c is light velocity and ν and λ are the frequency and wavelength of the photon. Due to the absorption of ultraviolet and visible light by an atom or molecule the electron present in it reaches to higher energy states. By absorbing light all molecules shows electronic excitation but in many cases for most of the atoms high energy radiation is required. Light remains in UV-Visible spectral region for molecules consisting of conjugated electron systems.

Principle of operation:

A light beam coming from UV or visible light source is divided into its component wavelengths using diffraction grating or a prism. By using partially silvered mirrors the monochromatic light beam is splitted into two equal intensity beams as shown in fig.3.6. Further the sample beam is passed through a cuvette which consists of solution which is to be examined in the transparent solvent. The reference is passed through an identical cuvette which contains only solvent. The intensities of both cuvettes are then measured and compared using electronic detectors. Intensity of sample beam is denoted by I. Whereas the intensity of reference beam which consist of no absorption or little absorption is incident light intensity (I_0). The spectrometer scans all of the component wavelengths within the range of 200-400 nm i. e. the UV region and 400-800 nm i. e. the visible region.

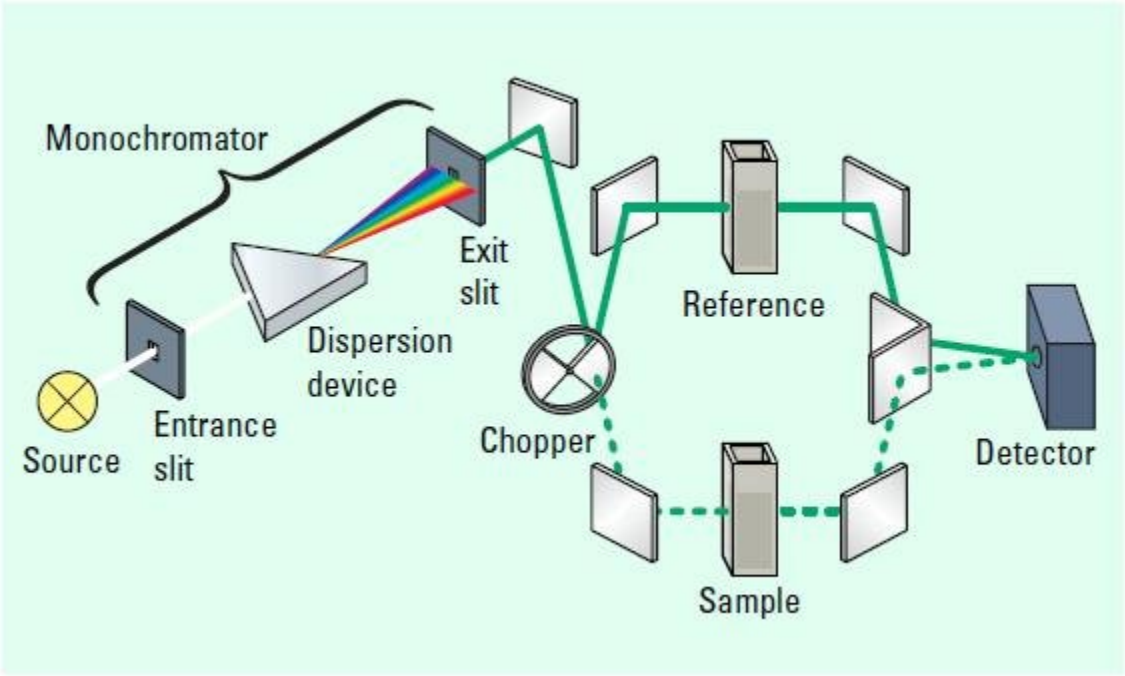

Fig.3.6. Schematic of UV-Visible spectrophotometer.

According to Beer-Lambert law the absorbance of a solution is directly proportional to concentration of absorbing species in the solution and path length. If the path length is fixed we can say that the technique can be used to detect the concentration of the absorber in the solution.

For a transparent solvent the intensity of light (I) which is transmitted from the solution of an absorbing chemical is related to its concentration by:

$$\mathbf{-Log\ I/I_0 = A = \epsilon \times b \times c}$$

Where b is cell path length in cm, A is absorbance, c is the solution concentration, I_o is the incident light intensity and ϵ is the molar absorptivity. We will use UV-Visible spectroscopy to determine melamine concentration in samples under investigations.

3.3.2. Dynamic Light Scattering (DLS)

DLS is a technique by which very small particle size which is in sub-micron region can be measured which is also known as Photon Correlation Spectroscopy or Quasi-Elastic Light Scattering. It is used for measuring brownian motion of particles and correlating it with the size of the particles. Brownian motion is defined as the random motion of particles within a solvent due to the bombardment of solvent molecules. It is the method which mainly used for studying the particle suspensions. As the particle size increases, the brownian motion decreases. Because when the particle sizes are smaller they can be easily "kicked" by the solvent molecules and thus their velocity increases. There is a term known as translational diffusion coefficient (D) which is mainly used to define Brownian motion of particles. Translational diffusion coefficient is mainly used to measure the hydrodynamic size of particles using the Stokes- Einstein equation:

$$\mathbf{d\ (H) = kT/3\pi\eta D}$$

Where k is Boltzmann's constant, η is viscosity, *d (H)* is hydrodynamic diameter, T is absolute temperature and D is translational diffusion coefficient. The diameter measured by this technique is the sphere diameter possessing similar translational diffusion coefficient as a particle has. This parameter is depends on the surface structure, the size of the particle "core" and the type and concentration of ions in the medium.

Working

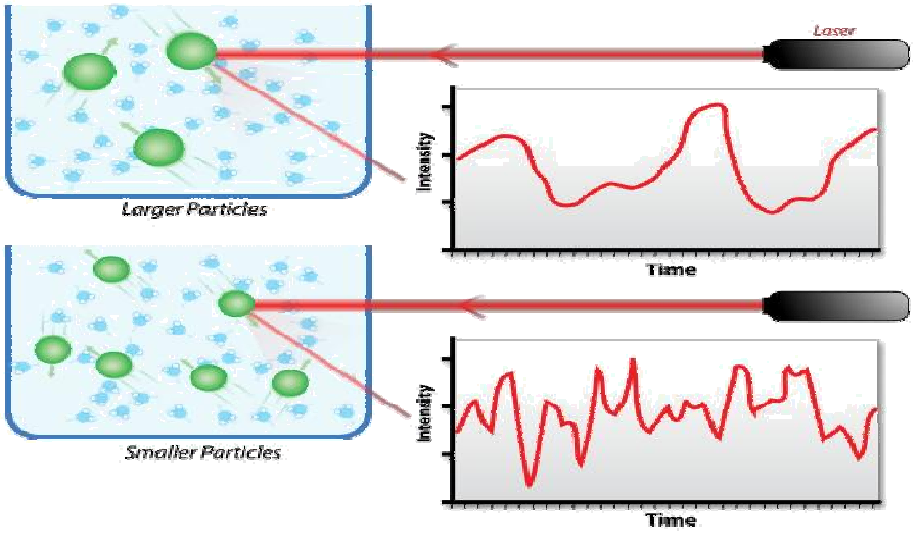

Fig.3.7. Schematic of scattering by small and large particles.

In dynamic light scattering the diffusing rate of the particles because of the Brownian motion is determined. Which can be done by measuring rate of change with the intensity of scattered light fluctuates which is detected by a proper optical arrangement. The schematic of light scattering by small and large particles is shown in fig.3.7.

The time intensity of the scattered light is going to be fluctuate with the random motion of particles in a liquid. The fluctuating signal is processed by the formation of autocorrelation function, C (t) where t being the time delay. With increasing time, correlation is lost and the function approaches to constant value of term B. When time is short correlation value is high. When the condition is in between these two cases the function decays exponentially for a monodisperse suspension of globular and rigid particles, it is given by:

$$C(t) = A\, e^{-2\Gamma t} + B$$

Here A is an optical constant used to determined the instrument design and Γ is a term which is

related to the fluctuations by:

$$\Gamma = D\, q^2$$

For calculating the value of q the index of refraction n is taken as 1.33, scattering angle θ as 90°, the wavelength of the laser light λ_0 as 0.635 μm of the suspending liquid. The equation which relates these quantities are given by:

$$q = (2\pi n/\lambda_0)\, 2Sin\,(\theta/2)$$

The translational diffusion coefficient (D) is calculated by QELS. It is a property of particles and macromolecules which posses much interest. QELS is the technique which is mainly used for measuring diffusion coefficients of micron sized particles. For calculating D only one assumption is required that is the particle shape should be globular no any other assumption relating to shape is required. All shapes have aspect ratio of greater than 5 untill it is needle i. e. in given first equation rotational diffusion term appears. Particle size is related to D for common simple shapes i. e. an ellipsoid, a sphere, random coil and a cylinder. Among all these, the assumption of spherical particles is most useful in a large number of cases. For a spherical shape:

$$D = k_B T / 3\pi \eta\,(t)\, d$$

Where η (t) is the viscosity of the liquid in which the particles are moving, T is the temperature in °K, k_B is Boltzmann's constant and d is the particle diameter. The main assumption for the equation is that the particles movement is independent of each other.

Thus the particle size measurement consists of:

- Autocorrelation function measurment.
- Determine of Γ by fitting the measured function.
- Calculating D if n, θ and Γ are given.
- Calculating particle diameter d if T and η are given.

3.3.3. Transmission electron microscopy (TEM)

Due to the limited image resolution of light microscopes transmission electron microscopes were developed which is imposed by the wavelength of visible light. By using this technique a user can examine very low level details as fine as a single column of atoms, which is many times smaller than the smallest resolvable object in the light microscope. In both categories of sciences i. e. physical and biological sciences transmission electron microscopy forms a major analysis method. At higher magnifications complex wave interaction modulates the intensity of the image, requiring expert analysis of observed images. The alternate modes in TEM give information about crystal orientation, chemical identity and morphology.

Principle

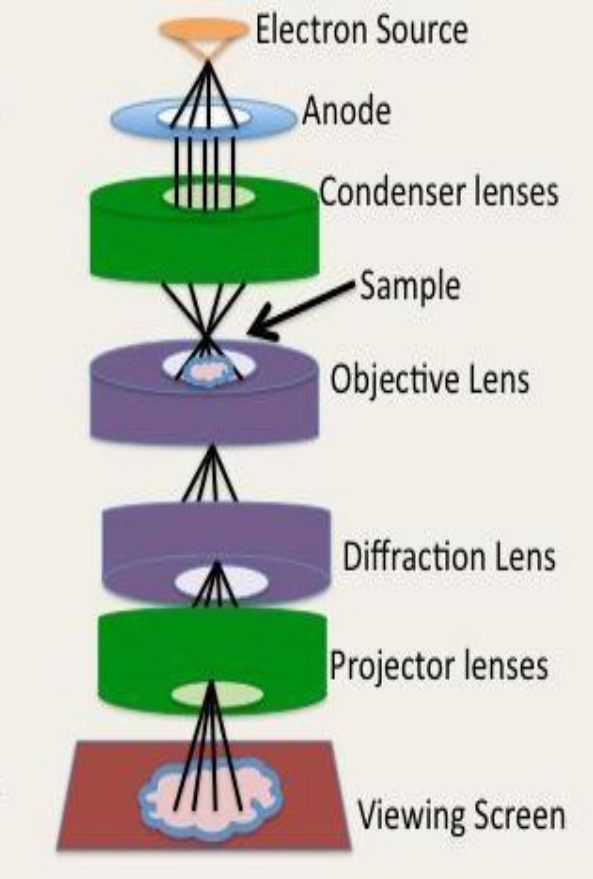

Fig. 3.8. Schematic view of Transmission Electron Microscope.

In this technique an electron beam is transmitted through an ultrathin specimen which interacts with the specimen as it passes through it. Due to the interaction of the electrons transmitted through the specimen an image is formed. The image which is formed is magnified and focused by an imaging device i. e. a fluorescent screen on a layer of photographic film or by a sensor i. e. CCD camera. TEM's are having a better resolution than light microscope due to the smaller de Broglie wavelength of electrons which is expressed by equation:

$$\lambda_{dB} = h/p$$

Where λ_{dB} is de Broglie wavelength, h is plank constant and p is the momentum of electrons.

Instrumentation

Transmission electron microscopy consists of many components as shown in figure 3.8 which include:

1. An electron emission source for generating electron streams and a vacuum system in which the electrons travel.
2. Electrostatic plates and electromagnetic lense assembly which allows the user to manipulate and guide the beam as per the requirement.
3. Small devices which are used for the motion, insertion and removal of specimens from the path of the beam.
4. For creating an image from the electrons that exitted by the system, imaging devices are used.

Applications

The instrument allows performing the following analysis:

1. Morphological analysis.
2. Structural analysis by electron diffraction.
3. EDS qualitative and semi-quantitative analysis.

4.1. Characterization of GNPs

4.1.1. UV-Visible spectral analysis

The UV-Visible absorption spectra of as-synthesized GNPs and aggregated GNPs were performed on shimadzu made double beam spectrophotometer (UV-2600). The measurements were performed in quartz cuvettes at room temperature. Surface plasmon resonance of GNPs (wine red) was observed at 524 nm (figure 4.1). The observation of single SPR peak indicates the formation of GNPs with spherical morphology.

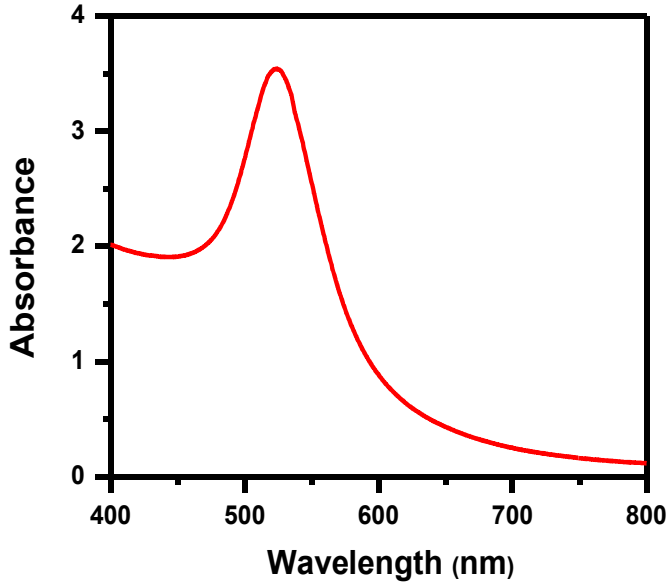

Fig. 4.1. UV-Visible spectra of as-synthesized GNPs showing SPR at 524 nm indicating the formation of spherical gold nanoparticles.

4.1.2. Dynamics Light Scattering (DLS) analysis

The hydrodynamic size of as-synthesized GNPs and aggregated GNPs after the addition of melamine is obtained on Brookhaven 90 plus particles size analyzer at different temperatures.

The hydrodynamic particle size is obtained by fitting the size distribution histogram with lognormal particle size distribution function. The hydrodynamic size of as-synthesized GNPs are recorded as shown in figure 4.2 which is 56.2 nm with a polydispersity of 0.338.

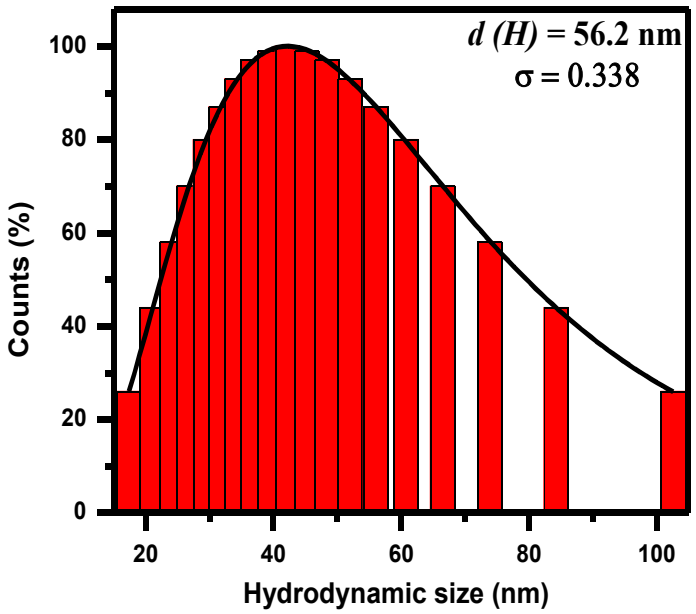

Fig.4.2. Hydrodynamic particle size distribution of as-synthesized GNPs fitted with log-normal particle size distribution.

4.1.3. Transmission Electron Microscopy (TEM) analysis

Transmission electron micrographs were taken on Philips transmission electron microscope (CM200) operated at an accelerating voltage of 200 kV. Selected area electron diffraction (SAED) pattern were also recorded. TEM image of GNPs and the average physical particle size of GNPs obtained from size distribution histogram are shown in figure 4.3. The average physical particle size of GNPs obtained is 19.66 nm. Five distinguished rings appear in the selected area electron diffraction (SAED) pattern of GNP's as shown in figure 4.4. These rings confirmed the polycrystalline nature of nanoparticles. The d-spacing of these rings as-observed by SAED are in good agreement with those found in JCPDS Card for Au Ref. No. 04-0784as shown in (table 4.1).

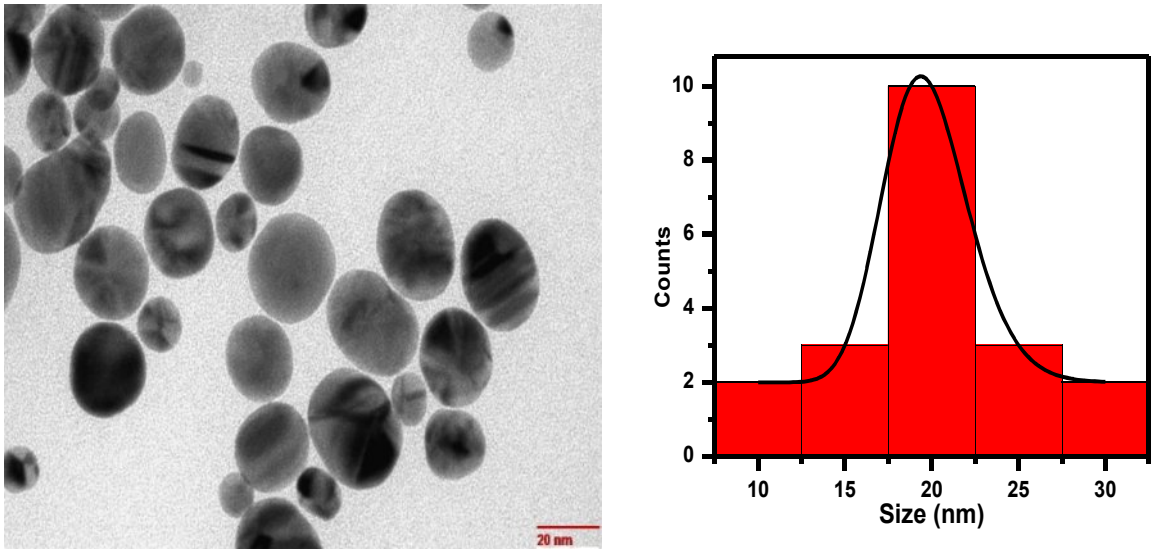

Fig.4.3. TEM image of as-synthesized GNPs and corresponding size distribution histogram.

Table 4.1: Comparision of d-spacing of GNPs obtained from SAED and reported in JCPDS data base.

(hkl) index	d-spacing (nm)	
	From SAED	From JCPDS (Ref. No. 04-0784)
(2 0 0)	2.00	2.03
(2 2 0)	1.41	1.44
(3 1 1)	1.3	1.23
(2 2 2)	1.11	1.17
(4 2 2)	8.33	8.32

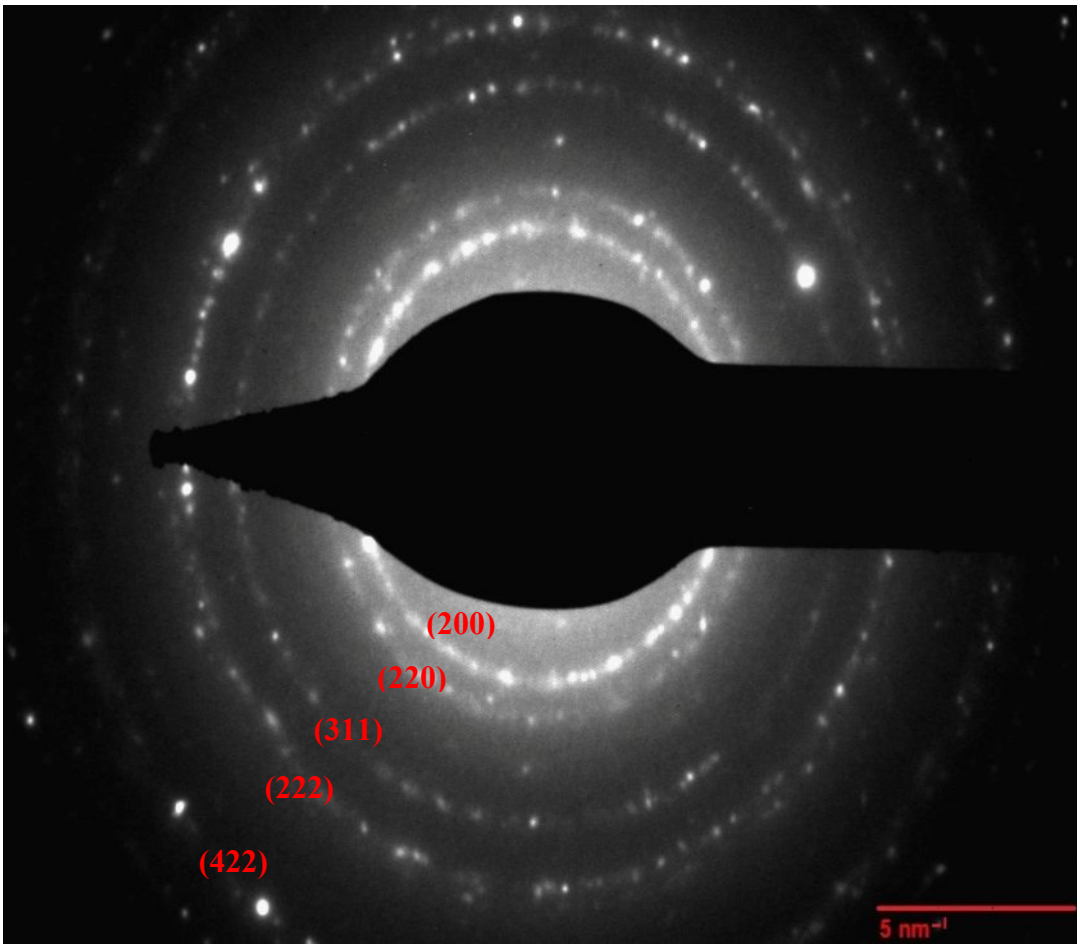

Fig.4.4. SAED pattern of as-synthesized GNPs.

4.2. Detection of Melamine using GNPs

After the addition of melamine, as-synthesized GNPs get aggregated due to which plasmon resonance peak shifts towards longer wavelength. The shift of plasmon peak is also showing visual color change from wine red to purple and further to blue (color for aggregated GNPs). GNPs gets aggregate because of the ring nitrogen and amine group of melamine, which strongly binds to the surface of citrate stabilized GNPs by ligand-exchange [28]. This ligand-exchange reduces the electrostatic repulsion between individual GNPs and finally caused their aggregation.

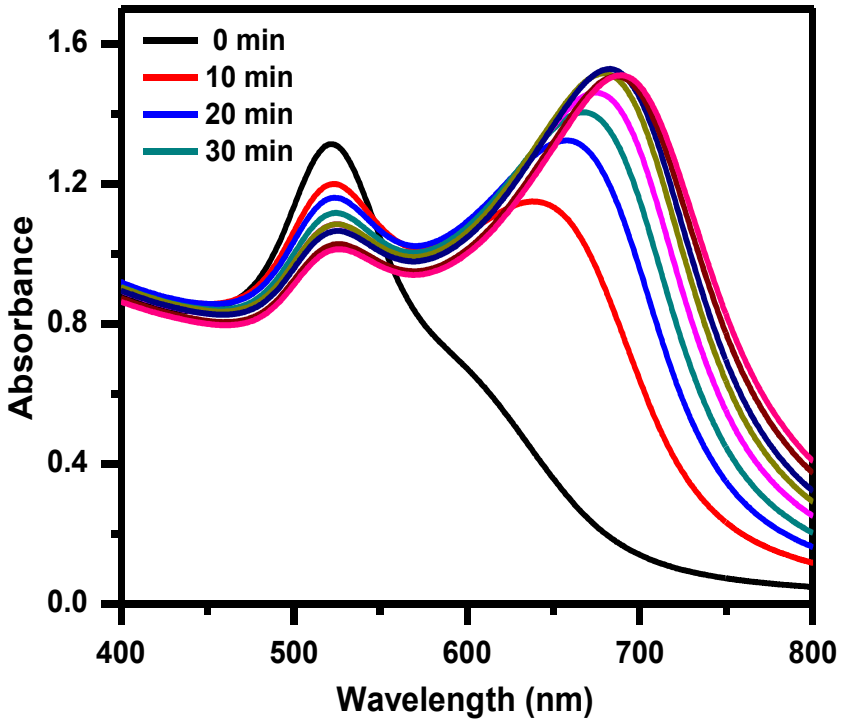

Fig.4.5. UV-Visible spectra of GNPs after reacting with 5 mM melamine.

Figure 4.5 shows the reaction kinetics of 5 mM melamine and GNPs. With time the surface plasmon resonance peak of wine red GNPs at 524 nm decreases and a new peak of aggregated GNPs at 650 nm is originated. After the completion of the reaction, peak at 524 nm is nearly suppressed and the new peak at 650 nm shifted to 680 nm due to increased aggregation.

Figure 4.6 shows the plot of A_{650}/A_{524} as a function of time. The absorbance ratio increases exponentially and saturates after 4000 seconds indicating the clustering of GNPs. This absorbance ratio measured for different melamine concentration is shown in figure 4.7. Increasing melamine concentration from makes a significant change in absorbance ratio for corresponding SPR peak. By taking intersection at maximum absorbance of all shifted peaks with respect to initial, absorbance ratio's are calculated and plotted against varying concentration.

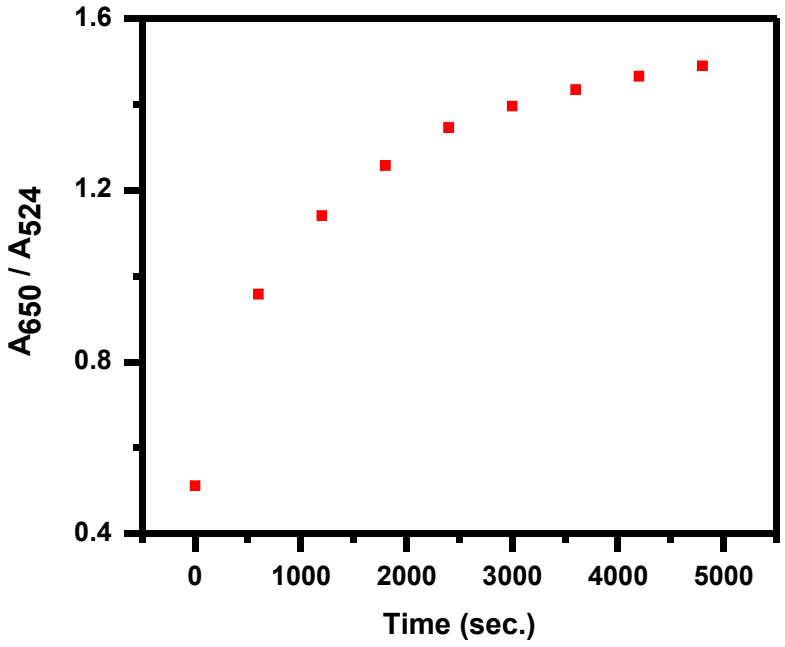

Fig.4.6. Absorption ratio of 5 mM melamine-GNPs as a function of time.

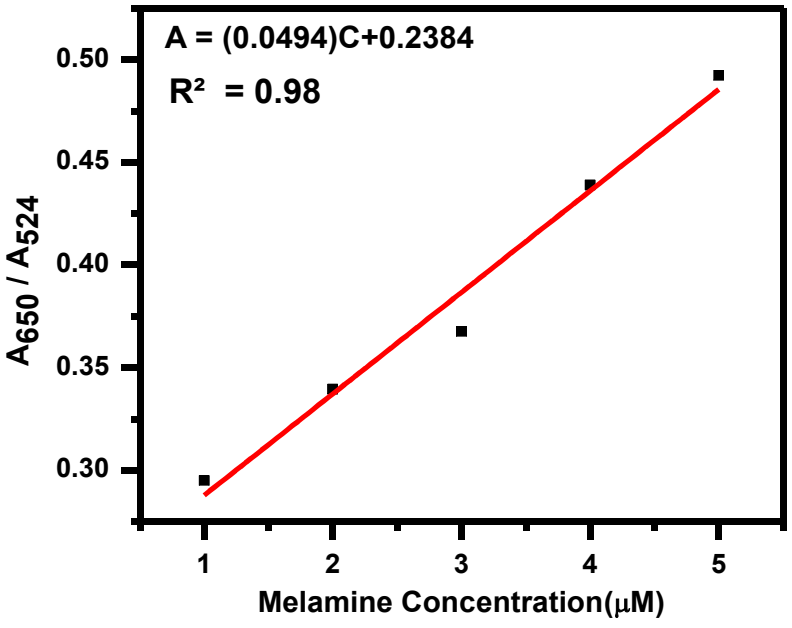

Fig.4.7. Plot of absorption ratio of GNPs-Melamine as a function of melamine concentration.

From this graph it can be conclude that with increasing concentration of melamine absorbance ratio increases. With increasing concentration of melamine color become purple to blue and further to dark blue [29]. So one can also estimate the concentration of melamine with naked eye observation. The variation in absorbance ratio with melamine concentration is linear. From the linear least square fitting, the melamine concentration in any milk based product can be determined by following the same procedure.

To study the aggregation behavior of GNPs aggregated size of GNPs after their exposure to 5 Mm melamine was determined at different time intervals at 15 °C, 20 °C and 25 ºC. The histogram are presented in figure 4.8, 4.9 and 4.10, respectively for 15 °C, 20 °C and 25 ºC. At 15 ºC, the hydrodynamic size increases from 10.04 nm to 89.40 nm after 300 minutes. Similarly at 20 ºC and 25 ºC, the hydrodynamic size of GNPs increases from 14.60 nm to 140.46 nm and 12.39 nm to 103.71 nm, respectively.

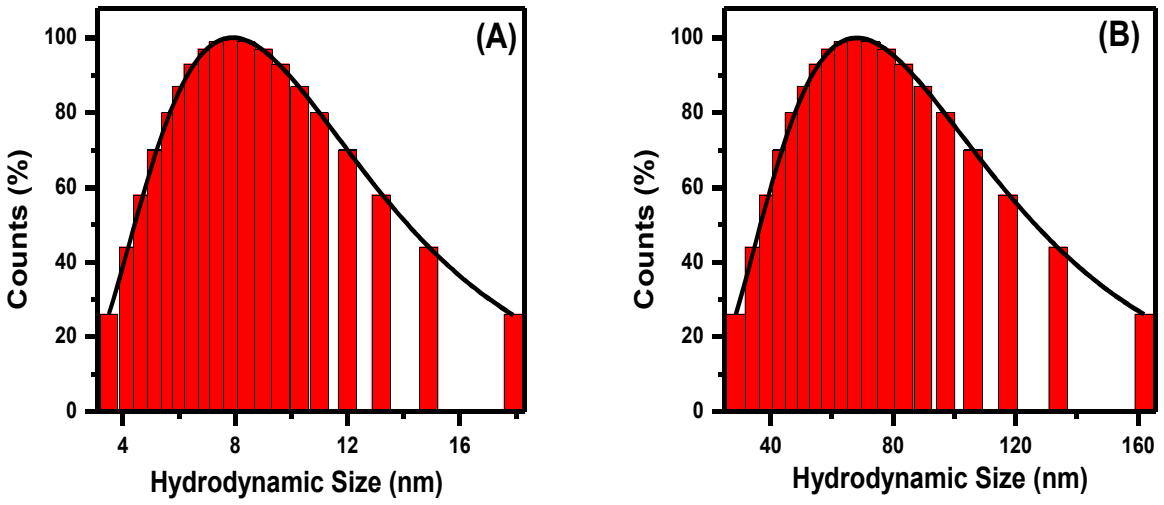

Fig.4.8. Hydrodynamic size distribution of GNPs after interacting with 5 mM melamine at 15 ºC after (A) 50 minutes and (B) 300 minutes.

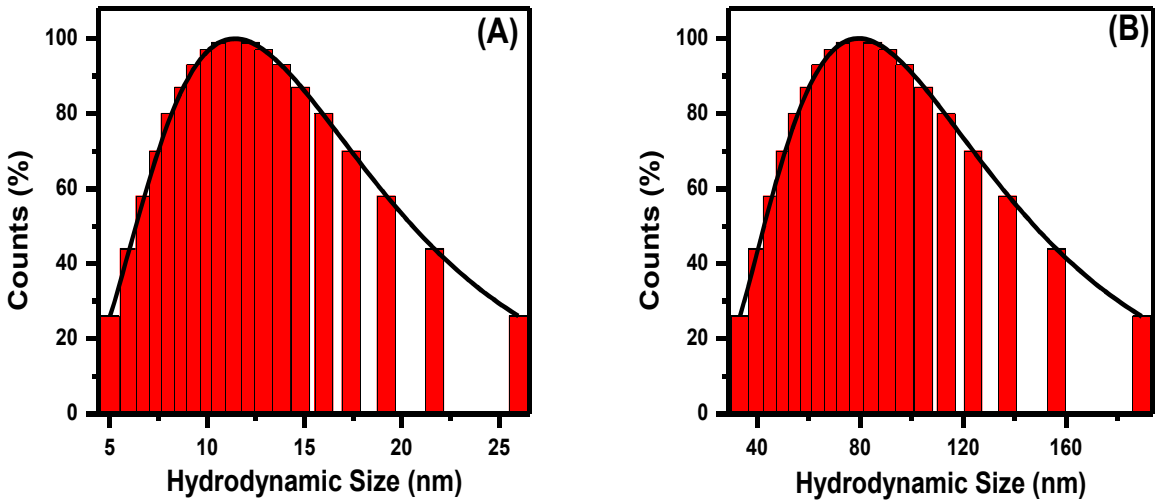

Fig.4.9. Hydrodynamic size distribution of GNPs after interacting with 5 mM melamine at 20 ºC after (A) 50 minutes and (B) 300minutes.

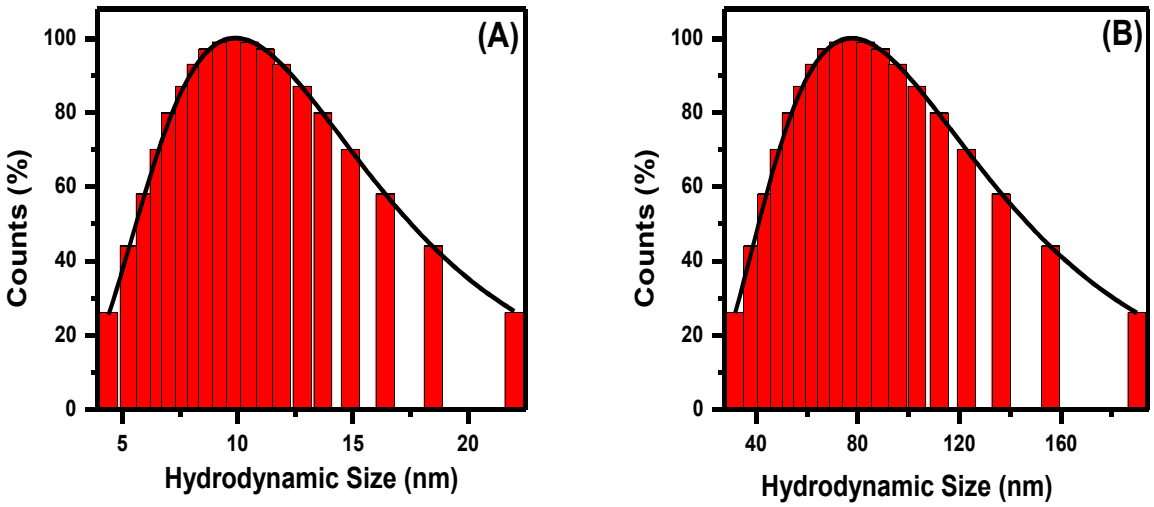

Fig.4.10. Hydrodynamic size distribution of GNPs after interacting with 5 mM melamine at 25 ºC after (A) 50 minutes and (B) 300 minutes.

Time variation of aggregate size of GNPs at different temperatures is also shown in figure 4.11. It can be observed that with time, hydrodynamic size of clusters increases linearly. Aggregation is more rapid at higher temperature as compared to lower temperature.

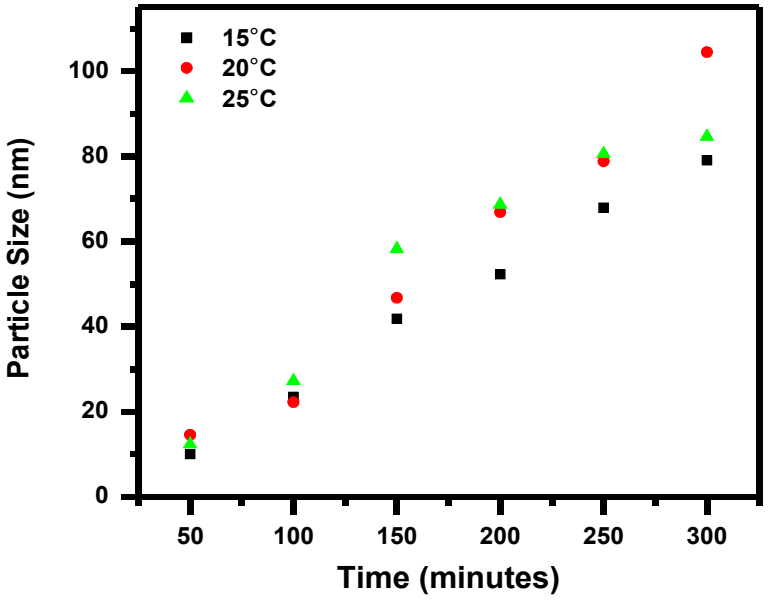

Fig.4.11. Time-variation in hydrodynamic size of GNPs upon their exposure to 5 mM melamine.

4.3 Detection of Melamine in Infant Formula Powder

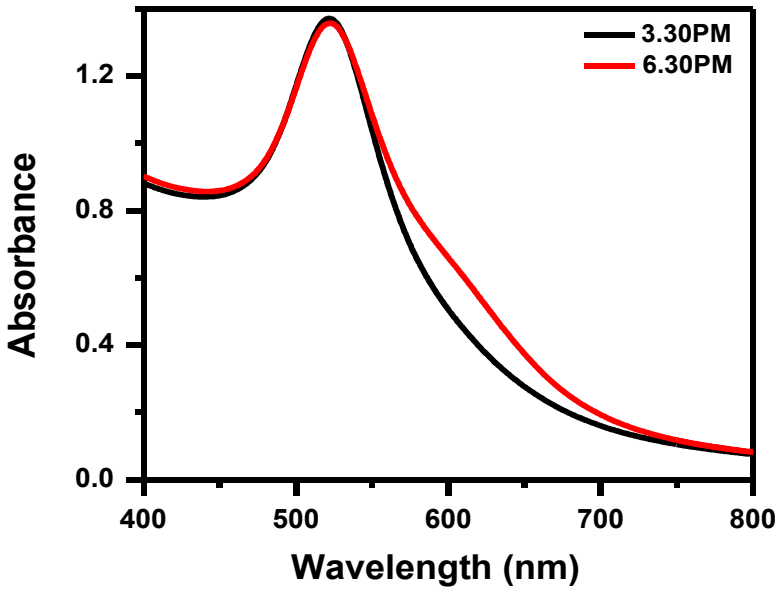

Fig.4.12. UV-Visible spectra of GNPs - infant formula powder.

Melamine induced aggregation of GNPs is used to determine the concentration of melamine in infant formula powder. Figure 4.12 shows the SPR peak shifting in sample containing infant formula powder and GNPs. When GNPs are added in infant formula powder the melamine present in the infant formula powder show a color change form wine red to deep red [31]. In case of infant formula powder the peak around 524 nm decreases slightly and a new peak at 630 nm develops that is due to the aggregation in the presence of melamine. Thus melamine can be identified in raw infant formula powder by measuring the absorption ratio at these two wavelengths. Using the calibration constants (Fig. 4.7), the concentration of melamine present in the infant formula powder was determined. In the tested sample, this concentration is 2.41 mM, which is higher than the permissible limit of detection (0.01 mM for infant formula powder by FDA) but the method still can be used due to its simplicity, reliability and low cost.

Conclusion

This dissertation highlights the use of SPR to examine food adulteration and contamination of melamine in milk based products. In this report a simple technique for the detection of melamine in infant formula powder by melamine induced color change of GNPs was demonstrated. Spherical GNPs are prepared and used as SPR probes to detect melamine in the sample under investigation. This study shows that melamine concentration as low as 2.41 mM can be detected. It can be further extended to identify the food adulteration in any milk based food products.

Scope for future

- A simple method to check food adulteration in milk based products has been developed. In this report the method is tested only on infant formula powder. However, this process can be used in principle to check melamine adulteration in any milk or milk based products.

- The role of physio-chemical properties and its correlation with detection limits can also be studied as an extension of this project.

- In the present study spherical gold nanoparticles are used as SPR probes. The same study should be conducted on other SPR probes with various morphologies to optimize the type of SPR probe suitable to detect particular adulteration.

- In general, the method demonstrated here can be extended to check adulteration in food products other than milk and milk based.

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