



Faculty of Sciences

Department of Chemistry and Forensic Sciences

**FABRICATION OF NOVEL NANOZYME BASED
COLORIMETRIC PROBES FOR THE RAPID, SENSITIVE
AND SELECTIVE SCREENING OF RESTRICTED
ADULTERANTS IN CONSUMER PRODUCTS: A CASE-
STUDY OF MERCURY IN COSMETICS AND
THIOCYANATE IN MILK**

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Abstract

This thesis presents novel nanomaterials known as nanozymes that were synthesized and applied as simple, easy-to-use colorimetric probes for the rapid, sensitive and selective detection of restricted low quality, cheap, and toxic substances usually added fraudulently to consumer products especially in food and cosmetics as adulterants; mercury in cosmetics and thiocyanate in milk. We synthesized nanomaterials known as nanozymes, which possess intrinsic catalytic activity toward certain colorimetric reactions. In the presence of model adulterants, mercury and thiocyanate, the catalytic-like activities of the nanozymes on these reactions were suppressed and the intensity of the accompanying color changes which were distinct to the naked eye were adulterant concentration dependent. The instant color changes allowed for the prepared materials to be explored as rapid colorimetric probes for the detection of restricted substances, mercury and thiocyanate in cosmetics and milk, respectively. Both materials exhibited excellent analytical performance as marked by very low limits of detection (LoDs); 2.34 nM for the mercury nanozyme and 2.89 μ M for the thiocyanate nanozyme which were well below acceptable levels of 4.99 μ M for mercury in cosmetics and 0.24 mM for thiocyanate in milk as set by the US Food and Drug Administration (FDA) and International Dairy Federation (IDF) respectively, and within the linear dynamic ranges of 0-10 nM for mercury and 2-50 μ M for thiocyanate. High recoveries ranging from 89.4 to 118.1 % for both nanozymes accompanied by excellent selectivities toward Hg^{2+} and SCN^- over potentially interfering species were obtained. The excellent characteristics of the developed nanozymes presented them as simple, fast, sensitive, selective, visual, equipment free point-of-need diagnostic tools of high throughput for adulterant analysis in consumer products.

Dedications



This thesis is dedicated to my husband, Thabo and children, Larona, Rorisang, and Agosi. You are my why! I also dedicate this thesis to young Oratile Mogapaesi. Girl, you have courageously and relentlessly gone after your dreams.

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Chapter 1

Introduction

1.1 Overview

In 2022, the global food and cosmetics markets were said to be worth \$8.66 trillion and \$534 billion and were expected to grow by 6.72% and 5.68% annually, respectively [1, 2]. As a result, there has been an exponential increase in the demand for food and cosmetic manufacturing ingredients. However, the rate of production of ingredients used to make finished food and cosmetic products is linear, and that does not match the exponential demand and market growth. To keep up, food and cosmetic producers have resorted to cheating, adulterating by either substituting an ingredient with a cheaper substance or removing a valuable component. Most of these adulterants that food and cosmetics manufacturers cheat with belong to a list of chemicals that a number of international environmental, food and health regulatory bodies prohibit for use as food and cosmetic ingredients [3]. Mercury and thiocyanate are common adulterants found in these lists.

Mercury is usually illegally added to cosmetic products, as a preservative [4] and to skin lightening products [5] as the active ingredient. Thiocyanate ion (SCN^-) on the other hand, may be fraudulently added to milk to enhance its shelf-life. These chemicals are toxic if used over prolonged periods or applied in high concentrations. For example, prolonged exposure to mercury results in respiratory, cardiovascular, gastrointestinal, hepatic, renal impairment, and reproductive effects [6]. It is also associated with elevated white blood cell count,

changes in auto-immune response, drop in vision and genotoxic effects [7]. Thiocyanate on the other hand, when ingested at higher levels causes anorexia, and mental status changes, including psychosis, seizures, tinnitus, and hyperreflexia [8]. It also acts as a goitrogen; a condition where excessively high concentrations of sodium thiocyanate in the blood hinder iodine uptake by the thyroid gland and induce goitre [9].

Given the potential adverse health effects of thiocyanate and mercury, governments and regulatory authorities around the world have introduced strict regulations on consumer products containing mercury [10] and thiocyanate [11]. The EU Cosmetics Regulation (EC) 1223/2009, prohibits mercury containing cosmetic products in the EU market except for thiomersal in eye products where a maximum concentration of 0,007% of mercury is permitted in eye makeup, mascara, and cleansing products [12]. The US Food and Drug Administration (FDA) under title 21 CFR 700.13, limits the quantity of mercury in cosmetic products to a trace quantity (1 ppm) unavoidable under good manufacturing practice except in products intended for use around the eye, in which the mercury regulatory limit is 65 ppm [13]. For regulation of thiocyanate, the Codex Alimentarius Commission (CAC)'s "Guidelines for the Preservation of Raw Milk by Use of the Lactoperoxidase system" recommends the addition of 14 mg of sodium thiocyanate (equal to 10 mg/kg of thiocyanate ion) per litre of raw milk for preservation [14]. Countries like India and China prohibit the use of thiocyanate to activate the LPS [11, 15].

To prevent the rejection of poor-quality products and increase shelf life, some unscrupulous manufacturers in the trade continue to add mercury and thiocyanate at concentrations above the stipulated regulatory levels in cosmetics and milk respectively. This cheating on consumers is not only dangerous to their health but is also fraudulent and is a form of adulteration. Adulteration in food refers to the addition of a poisonous or deleterious non-food item to increase the quantity of raw or prepared food which may render it injurious to health [16]. According to the US Federation of Food, Drugs and Cosmetics act title 21 chapter 9 subsection VI code 361a, a cosmetic product may be deemed adulterated if it bears or contains any poisonous or deleterious substance which may render it injurious to users under the

conditions of use prescribed in the labelling thereof, or under such conditions of use as are customary or usual [17]. The presence of adulterants in foodstuff and cosmetics may be deliberate or unintended. The unintended addition of adulterants may occur due to carelessness, poor hygiene and handling during processing, storage, transportation [18], and addition of harmful residues from packaging [19]. Intentional contamination (adulteration) involves the deliberate addition, substitution, tampering, misrepresentation of a product or its ingredients, false or misleading statements made about a product to heighten its appearance and quality for unfair economic gain [16].

To curb such acts of adulteration and protect consumers, it is necessary to employ highly sensitive, accurate, timeous, and selective analytical methods for adulterants detection. To date, a plethora of analytical instruments have been reported for the analysis of thiocyanate and mercury in various matrices. Thiocyanate has been analyzed using sophisticated, sensitive hyphenated instrumentation including ion chromatograph (IC) with a photodiode array detector (DAD) [20], conductivity detector [21, 22], mass spectrometer either in single state [23] or tandem [24], capillary electrophoresis (CE) with ultraviolet (UV) detection [25–27], high-performance liquid chromatograph (HPLC) using ultraviolet detection [28], electrochemical detection [29] or coupled with various types of mass spectrometers [30, 31], and gas chromatograph (GC) using electron capture detection (ECD) [32, 33], flame ionization detector (FID) or a flame thermionic detector (FTD) [34] and mass spectrometer [35]. Mercury is generally determined using hydride generation atomic absorption spectroscopy (HG-AAS) [36], cold vapor atomic absorption spectroscopy (CV-AAS) [37–39], inductively coupled plasma mass spectrometry (ICP-MS) [40–43] and most recently, microwave plasma atomic emission spectrometry (MP-AES) [44]. These contemporary analytical techniques have the sensitivity and accuracy required for adulterants detection and quantification [45]. They are however limited by expensive and sophisticated equipment that require professional personnel [46]. Additionally, the operation of some of these equipment is costly [47]. Consequent to the increasing number of adulteration cases and limitations mentioned, it is urgent to develop analytical tools, devices, methods, protocols, strategies that employ novel, smart functional materials that are easy to develop, cheap, sensitive and can assist in achieving rapid,

easy to interpret, precise and accurate analysis or screening especially at ports of entry for a landlocked country like Botswana that heavily relies on importation of goods. Recently, nanozymes have been identified as such materials.

Nanozymes are nanomaterials with intrinsic enzyme-like characteristics [48] and are known to catalyze various types of reactions including reduction reactions, oxidation reactions and polymerization reactions [49]. They have attracted attention because of their use for on-site analysis, visualization, simplicity, high throughput, sensitivity, and no requirement for skillful technicians. [50]. In the presence of certain contaminants, the catalytic like activity of nanozymes on these reactions are either selectively enhanced [51] or inhibited [52]. In this study we use the inhibition of catalytic like activities of nanozymes for the analysis of mercury and thiocyanate.

1.2 Statement of the Problem

Globally, monitoring of restricted substances in consumer products is limited. Most analysis of the adulterants is performed in centralized laboratories and only a limited number of samples can be tested due to long preparation, operation time and low throughput. Moreover, conventional methods of analysis are limited by expensive, sophisticated instrumentation that require professional personnel and long analysis times even though they offer the sensitivity and accuracy required for trace contaminant analysis however are limited by expensive, sophisticated instrumentation that require professional personnel and long analysis times.

1.3 Aim and Objectives

The main aim of this study was to develop novel custom made nanozyme probes for the selective screening of mercury in cosmetics and thiocyanate in foodstuff.

1.3.1 Specific Objectives

The specific objectives of this study were to:

- synthesize Au@ZnO/Fe₃O₄ hybrid material and silver nanoparticles with catalytic-like activity toward reduction of 4-NP and oxidation of TMB, respectively.
- assess the effect of Hg²⁺ and SCN⁻ towards the catalytic activity of the synthesized particles and hence develop colorimetric probes for the screening of Hg²⁺ and SCN⁻ in a cosmetic product and milk respectively.
- evaluate the efficacy of the developed probes in terms of sensitivity, selectivity and recoveries.
- apply the prepared nanozymes as probes for the screening of mercury and thiocyanate in a cosmetic product and milk, respectively.

1.4 Significance of the Study

Adulteration in consumer products is a global concern. To ensure consumer protection, regular monitoring of consumer products is required. However, current methods for detection of adulterants require elaborate sample preparation steps prior to analysis, involving high-end instrumentation that are difficult to operate and time consuming. Therefore, considerable interest has emerged in developing sensitive, selective and rapid methods with a potential for on-site analysis of trace adulterants in consumer products. Through this work we successfully employed a novel method to decorate ZnO/Fe₃O₄ with AuNPs. Furthermore, to the best of our knowledge, we are the first to employ a green reducing agent (marula leaf extract) in the synthesis of enzyme-mimetics for the detection of thiocyanate based on their oxidase-like activity with oxygen as a green oxidant. All other nanozymes for the detection of thiocyanate were synthesized using toxic chemical reducing agents and the detection of

thiocyanate was based on their peroxidase-like activities which use destructive and unstable H_2O_2 . Through this work we offer green sustainable alternatives for the synthesis of the nanozymes for the detection of thiocyanate and its detection. The UN sustainable development goal 3 and the Africa Agenda 2063 goal 3 aspire to ensure health and well-being for all. Similarly, one of the objectives of Botswana's second pillar of vision 2036 on human and social development is that Botswana will live long and healthy lives. This work contributes to these goals and pillar. The availability of rapid colorimetric, sensitive and selective analysis tools for consumer product adulterants detection, which we develop in this work, is a must for ensuring the good health and well-being of consumers.

1.4.1 Contributions

- **O. Semong**, B.S. Batlokwa, Rapid Colorimetric Detection of Hg (II) Based on Hg (II) Induced Suppressed Enzyme-like Reduction Of 4-Nitrophenol by Au@ZnO/Fe₃O₄ in a Cosmetic Skin Product. Nanomaterials and Nanotechnology. (under review)
- **O. Semong**, B.S. Batlokwa, Morula Leaf Extract Mediated Synthesis of Silver Nanoparticles and their Application in the Analysis of SCN⁻ Based on Inhibition of the Nanozyme Activity on 3,3',5,5' Tetramethylbenzidine (TMB) by the Silver Nanoparticles. J Food Chem Nanotechnol., 2022, 8(4): 192-200.
- B.S. Batlokwa, **O. Semong**, R. Majors, N. Torto, A Highly Sensitive Custom-Synthesized Molecularly Imprinted Sorbent for the Selective Extraction of Aflatoxins from Nut Samples and Clean-Up Prior to Analysis by HPLC-FLD. Adv Res Chem & App Sci., 2020, 2:2.

Chapter 2

Literature review

2.1 Overview

In this chapter we review current literature on the adulteration of food and cosmetics and the application of nanozymes in the detection of mercury and thiocyanate. We first discuss types of adulteration, the challenges in monitoring of consumer products, and common adulterants in food and cosmetics. Furthermore, we focus on mercury and thiocyanate as adulterants in cosmetics and milk; their function, possible adverse health effects, their regulation and conventional methods for their analysis and their limitation. We then explore the potential for application of nanozymes in the detection of mercury and thiocyanate in varying matrices, focusing on classes of nanozymes, their design and construction, characterization using different techniques and their underlying mechanisms in the detection of mercury and thiocyanate.

2.2 Adulteration of Foodstuff and Cosmetics With Restricted and Prohibited Substances

Generally, adulteration is the cheating of consumers either intentionally or unintentionally by substituting an ingredient with an inferior substance or removing a valuable component [18]. This usually results in lowering of the quality of the product. According to Federal Food, Drug and Cosmetic Act (FFDCA), the primary food safety law administered by the

Food and Drug Administration (FDA), food can be declared adulterated if;

- a) a substance is added which is injurious to health,
- b) a cheaper or inferior quality item is added to food,
- c) any valuable constituent is extracted from the main food article,
- d) the quality of food is below the standards,
- e) any substance has been added to increase the bulk weight and make it appear more valuable [53].

In cosmetics, a product is considered adulterated if it contains a substance that makes it harmful to consumers under customary conditions of use, such as;

- a) if it contains a filthy, putrid, or decomposed substance;
- b) if it is manufactured or held under unsanitary conditions where it may become contaminated;
- c) if it contains a non-permitted color additive (except where it is a dye). It should be noted that coal-tar hair dyes indicating that a "patch-test" must be performed before use are exempted from the adulteration provision even if they are irritating to the skin or are otherwise harmful to the human body. However, eyelash and eyebrow dyes are not included in this exemption. The FDA approves or disapproves all dyes used in eyelash and eyebrow dye products [54].

Adulteration may be intentional or unintentional. Unintentional adulteration is the inclusion of unwanted substances due to ignorance, carelessness or lack of proper facilities and hygiene during processing [18]. On the other hand, intentional adulteration refers to the deliberate debasing of a product by counterfeiting a genuine commodity or substituting a superior article with an inferior one through the addition of prohibited substances, adding

restricted substances at concentrations above the regulatory limit, or the unapproved addition of ingredients inapplicable to the type of product [16], which is the focus of this work. Intentional adulteration is usually perpetrated by producers for; economic gain (economically motivated adulteration (EMA)), disgruntled employees revenging on their employers or coworkers, terrorists with the motive to cause harm to civilians and/or disrupt the social, economic, or political security of those they perceive as their enemy. This is referred to as food or cosmetics terrorism.

2.2.1 Economically Motivated Adulteration (EMA) in Food and Cosmetics

EMA is the fraudulent, intentional substitution or addition of a substance to a product for the purpose of increasing its apparent value or reducing its cost of production, for economic gain [55]. Certain trends and issues such as consumer demands, high product demand and global supply chains create new opportunities and motivations for economically motivated adulteration [56]. Food and cosmetics production are driven by consumer trends, whether it is a flavor trend e.g., addition of sriracha-flavored almonds, or a perceived health benefit e.g., paraben/sulphate free cosmetics and lactose/gluten free foods. Furthermore, recently consumers are increasingly asking questions about the history of the food they consume such as, agricultural practices of farmers and their sustainability.

There is a current obsession with physical appearance which has sent the food and cosmetic industries into an overdrive. People prefer meal replacements such as energy bars and diet shakes to keep slim [57]. While current beauty trends are no longer about bold makeup but healthy, moisturized, younger-looking skin, women are not under pressure to look younger, increasing the demand for skin care products [58]. Consequently, consumers are willing to pay additional costs for their demands, making it a lucrative business for both legitimate producers and fraudsters. Globalization has created gaps in safety in the food and cosmetics supply chain [59]. Global supply has grown in volume and complexity [60]. With an increasingly long and complex global supply chain comes the challenge of product traceability, over-stretched and limited oversight over product quality and safety to address the

increasing number of cheating manufacturers who commit economic fraud [55].

EMA has far-reaching implications. The health risks associated with adulteration could be instantaneous, for instance a single exposure to an adulterated product may lead to an allergic reaction. In some cases, prolonged exposure to an adulterant may lead to chronic health effects as a result of adulterant build-up in the body over long periods due to ingestion or dermal contact of low doses of such adulterants [61]. In food, where consumers are denied the full nutritional benefits associated with a certain food or food product due to addition, substitution or modification, consumers may suffer from malnutrition [62]. In addition, adulteration has financial implications and weakens consumer trust in manufacturers [63]. The estimated cost to the food and cosmetics industries is in billions of dollars annually. In addition the credibility producers and distributors is damaged [64]. EMA undermines profit margins for legitimate producers and creates an enormous financial liability for regulatory bodies [65].

2.2.1.1 EMA in Food

EMA in food is usually referred to as food fraud. The term food fraud encompasses the deliberate and intentional substitution, addition, tampering, or misrepresentation of food, food ingredients, or food packaging; or false and misleading statements made about a product with the goal of inflating its financial value or reducing production costs of such products [66]. Olive oil, coffee, honey, fruit juice, spices, and milk are said to be the seven foods most likely to be targeted for food fraud [53].

Foods that are said pure, wholesome, natural, and have high health benefits such as olive oil, coffee and honey are high value foods and are thus vulnerable to adulteration due to their strong economic incentive [67]. Adulterants are added to counter high prices, product scarcity or lower production costs by substituting high value foods with their low value counterparts [68]. For example, in the olive oil industry, the advertising of virgin olive oil as extra virgin olive oil (EVOO), and mixtures of other less valuable vegetable oils (blends of sunflower, corn, palm, rapeseed oil, etc with olive oil) as pure olive oil is common [69].

Similarly, high value specialty coffee beans are mixed with low value commodity coffees [70]. In the sweetener industry, honey is diluted with other less expensive sweeteners such as corn, cane, and beet syrups [71]. The high disparity in the prices of high value foods compared to other foods, can lead to significant economic advantage for deceitful producers [72].

In addition to high economic value, their compositional and nutritional characteristics, put high value products at high risk of adulteration [69]. The chemical make-up and sensory properties of twigs, roots, legumes, and other roasted grains such as corn, and rice do not have a drastic effect on those of coffee therefore, these substances have been added as adulterants in coffee. These adulterants however, do not contain caffeine, the main active ingredient in coffee, which is beneficial to human health [73]. Similarly, high quality honey is in demand, this is because it contains a wide range of beneficial phytochemicals [74, 75], most of which are absent in its common adulterants such as corn, cane, and beet syrups. Although seemingly harmless, adulterating these high-quality foods with their less healthy counterparts deprives consumers of value for money as well as their nutritive benefits that may sometimes cause adverse health effects. Additional health issues may also occur if an adulterant has an allergenic effect on some consumers [76].

To protect consumers, high value foods are now heavily regulated. Unfortunately fraudsters are adapting and developing new sophisticated ways of adulteration. For instance, to make edible oils “undetectable” when mixed with olive oil some manufacturers desterolize them [77]. Moreover, vegetable oils with similar fatty acids and sterols composition to olive oil are added as adulterants [78]. In some cases, 100 % fruit juice producers add trace concentrations of artificial sweeteners that would not be easily detectable or even be easy to differentiate from the natural glucose and fructose present as part of the fruit [79]. In addition to adulterating 100 % fruit juices with artificial sweeteners, colorants are often added to improve the colour score of fruit juices, hence their final grade level and value are enhanced [80]. In spices, a prohibited dye Sudan red, a known carcinogen, has been added to chilli powder, to enhance its color and present false sense of quality [81]. Yellow chalk powder has been

added to turmeric spice to increase its bulk weight [82]. Increasing bulk weight as a form of adulteration is also common in milk, where milk is watered down to increase its volume [83]. Also, beneficial fats are often removed from milk. To mask these forms of adulteration and promote the level of essential nutrients in milk, adulterants such as soya milk, starch, groundnut milk and wheat flour have been added and even more detrimental to human health, toxic substances such as detergents, urea, and melamine have been added to milk [53]. In some cases, addition of chemical substances intended for the good purpose of lowering the growth of microbes and increasing product shelf life in food such as milk, are often adulterated by addition of more than required [84]. Examples of such substances include hydrogen peroxide, formaldehyde, hypochlorite, salicylic acid, potassium dichromate, formalin, antibiotics and thiocyanate which are all toxic to humans if excessively used [85, 86]. Of these, thiocyanate is one of the most highly adulterated as it is the most commonly used for decreasing microbial growth in milk these days, thus our choice to study it in this thesis.

Thiocyanate ion, is a natural component of the lactoperoxidase milk preservation system (LPS) [15]. Lactoperoxidase system a natural antibacterial system in milk which is activated by increasing the concentration of thiocyanate in milk. In the system, thiocyanate ions are oxidised by the lactoperoxidase enzyme in the presence of hydrogen peroxide (H_2O_2). The major product of this reaction is hypothiocyanous acid (HOSCN). At pH 6.7, HOSCN is broken down and exists predominantly as hypothiocyanate ions (OSCN^-) [87]. OSCN^- reacts specifically with free sulphydryl groups ($-\text{SH}$). This results in inactivation of numerous important metabolic bacterial enzymes, hence, inhibiting their metabolism and ability to multiply, therefore prolonging the shelf life of milk [14]. SCN^- 's inherent concentration in milk is about 5–8.5 mg/L [9]. To activate the lactoperoxidase system, 20 mg/L of thiocyanate is needed [88]. In countries where adequate refrigeration equipment is not accessible, and where it is permitted by regulation, the LPS may be artificially activated by augmenting the thiocyanate ion using sodium percarbonate or sodium thiocyanate to temporarily inhibit pathogenic microorganisms and increase storage stability of milk [89]. Food and Agriculture Organization (FAO) recommends that when used as intended, the level of thiocyanate added to milk for treatment be within the natural variation in thiocyanate found in milk and

the levels of thiocyanate in LPS treated milk must not present a food safety concern. Therefore, the Codex Alimentarius Commission (CAC)'s "Guidelines for the Preservation of Raw Milk by Use of the Lactoperoxidase system" recommends the addition of 14 mg of sodium thiocyanate (equal to 10 mg/kg of thiocyanate ion) per liter of raw milk for preservation [14]. Conversely, some researchers state that lower doses (4.8–6.4 mg/L) of thiocyanate competitively inhibit iodide uptake by the sodium-iodide symporter in the thyroid gland and induce goitre [9].

For this reason, in countries like India and China, activating the LPS by adding thiocyanate is not permitted by the food regulatory authorities [11, 15]. Regardless, to prevent the rejection of poor-quality products and increase shelf life, some producers add thiocyanate at concentrations above the stipulated regulatory limits. However, if the thiocyanate concentration in plasma is higher than 200 mg/L, fatalities may occur [25]. When ingested at such high levels SCN^- is also known to cause anorexia, and mental status changes, including psychosis, seizures, tinnitus, and hyperreflexia [8].

2.2.1.2 EMA in Cosmetics

Adulteration of cosmetic ingredients to lower the cost of production is a common problem in the cosmetic industry. In addition to this, cosmetics are adulterated to improve the short-term efficacy of the products. Common adulterants in cosmetics include pharmacologically active substances such as antibiotics, corticosteroids, and sexual hormones (oestrogens, progestogens, androgen), nitromusk fragrances, phthalates, preservatives, and whitening agents [90].

Article I from CEE Cosmetic Directive 76/768/EEC, aiming at regulating the production of cosmetic products, defined exactly what can be considered a cosmetic product: "For the purposes of this law, a "cosmetic product" shall mean any substance or preparation other than medicines, to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view, exclusively or mainly to cleaning them, perfuming

them, protecting them, keeping them in good condition, changing their appearance and/or correcting body odour" [91]. By design, cosmetic products have no therapeutic purpose and must not claim any therapeutic action and therefore should not contain pharmacologically active substances. With long-term use, high dosage, and especially if used without medical supervision cosmetic products containing pharmacologically active substances such as antibiotics, corticosteroids, and sexual hormones present harmful side effects [92].

To achieve functions such as whitening, eliminating freckles, removing acne, antibiotics are usually added to cosmetics [93]. Quinolone antibiotics have been widely used in anti-acne cosmetics [94]. However, recent studies reported that quinolone antibiotics showed potential genotoxicity [95]. In addition, quinolones have been reported to cause neonatal alterations in articulation cartilages, bone growth, and tendons, both in humans and animals [96]. Therefore, antibiotics are prohibited in anti-acne cosmetics, according to the Chinese Hygienic Standard for Cosmetics (2007) and Council Directive 76/768/EEC of the European Union (EU) and its amendment [94]. Unfortunately, some illegal anti-acne cosmetic preparations in the black market have been found to contain antibiotics. Jin et. al. determined antibiotics in anti-acne cosmetics and found metronidazole in a few anti-acne products they had obtained from the black-market [94].

Corticosteroids are known to be highly effective drugs widely used in dermatology for the treatment of inflammatory diseases [97]. They are used for the treatment of skin disorders such as psoriasis, dermatoses, and eczema [25]. Their use in cosmetic products is however prohibited by the Directive 76/768/CEE, enforced in Italy on 11/10/86 by law no. 713, and they are also prohibited in several countries outside the European borders [98]. Yet some manufacturers advertise their cosmetic products as being effective for the improvement of red, itchy, flaky skin caused by eczema, seborrhoea and psoriasis and illegally add corticosteroids without indicating such on the label [92]. Prolonged therapy with corticosteroids preparations may result in adverse effects like skin atrophy, cutaneous reactivity and some systematic side effects, hypertension, diabetes mellitus, osteoporosis, allergic contact dermatitis, and Cushing's syndrome among others [97]. It is also worth noting that despite their

known adverse side effects corticosteroids are still considered legal cosmetic ingredients in several countries [99].

Skin whitening cosmetics are usually used to even-out skin tone usually on the facial area and the back of the hands, but sometimes they can be used more extensively over larger areas of skin [100]. Skin bleaching cosmetics contain whitening agents such as kojic acid, salicylic acid, nicotinamide, arbutin, hydroquinone, corticosteroids, retinoic acid, and mercury [98]. However, chronic side effects of hydroquinone include ochronosis, nail discolouration, conjunctival melanosis, and corneal degeneration [98]. The teratogenic nature of retinoic acid has been demonstrated [101]. As already mentioned, side effects of topical corticosteroids include Cushing's syndrome, hyperglycemia, and menstrual irregularities, while dermatologic side effects include skin atrophy, striae, and acne [102] while mercury is most predominantly known to be nephrotoxic and neurotoxic [103]. Due to their obvious side effects, these compounds are prohibited for use as cosmetic ingredients in skin lightening cosmetics both in the EU and several other countries outside the European [104]. Compared to legal skin lightening active agents, hydroquinone, corticosteroids, retinoic acid, and mercury are highly efficient. Therefore, despite their legal ban, they are usually adulterated in skin bleaching cosmetics [98].

Adding sexual hormones to cosmetics has several advantages. They promote hair growth, eliminate wrinkles, increase skin elasticity, and prevent skin from aging [105]. Oral contraceptives containing hormones such as progesterone and estrogen are used to fight biochemical signs of hyperandrogenism (hirsutism, acne, and male-pattern hair loss) [91]. In addition, the simultaneous presence of phosphodiesterase (PDE-5) inhibitors (sildenafil, vardenafil and tadalafil) with topical testosterone and anaesthetics, used in pharmacological treatment of premature ejaculation is believed to be effective in treating erectile dysfunction in aged men [106, 107]. In China, the use of sexual hormones in cosmetics has been forbidden, including male and female hormones, and progestogen (GB7916-87) [105]. The presence of sexual hormones and anaesthetics in cosmetic creams is expressly forbidden by (Annex II), Article I from CEE Cosmetic Directive 76/768/EEC [108]. In addition, even though it is not men-

tioned in the above reported Annex II, PDE-5 inhibitors should not be added to cosmetic products, since they are also pharmacologically active compounds requiring medical prescription even when present in topical preparations.

However, recently, the Italian anti-adulteration and safety bureau (NAS) seized several illegal cosmetic creams sold through internet web sites or illegal circuits believed to contain pharmacologically active substances reported above. Being preparations for topical use, they are seen as “safer” by consumers than capsules and tablets. Nonetheless, the long-term use of hormones in cosmetics can cause metabolism disorder and cancer [105]. PDE-5 inhibitors cause headache, facial flushing, nasal congestion, dyspepsia, and back pain [109]. Visual events including nonarthritic anterior ischaemic optic neuropathy have also been reported and caution is advised in prescribing PDE-5 inhibitors to patients who have had a myocardial infarction or stroke, those who have resting hypotension or hypertension, unstable angina, and cardiac failure, as there is no controlled clinical data in patients with these types of adverse events [110].

Phthalates (phthalic acid esters, PAEs), especially diethyl phthalate, on the other hand, are commonly used as solvents and odourless diluents in cosmetics and perfumes [111]. They are used primarily as plasticizers in cosmetic products such as nail polishes to reduce cracking by making them less brittle, and in hair sprays to help prevent stiffness by allowing them to form a flexible film on the hair [112]. They are also used as perfume fixatives in various other products [113]. In recent years, they have attracted great public attention because of their suspected carcinogenic and estrogenic properties [111]. According to some studies, phthalates are associated with decreased thyroid function and reproductive hormone in the general population and pregnant women [114]. Due to their potential risks for human health and the environment, several of them have been included in the priority list of pollutants by different national and supranational organizations [115]. Among the principal phthalates used in cosmetics, eight phthalate esters including di-n-butyl phthalate (DBP), di-iso-butyl phthalate (DIBP), butyl benzyl phthalate (BzBP), bis (2-ethylhexyl) phthalate (DEHP), di-n-octylphthalate (DNOP), di-isodecyl phthalate, di-n-pentyl phthalate, and di-isononyl ph-

thalate are now part of the list of chemicals of concern by the United States Environmental Protection Agency (USEPA) [116]. DBP, DEHP, DMEP and di n-pentyl phthalate (DPP) are prohibited by the European Union (EU) [117]. Nonetheless, essential oils used as fragrances in cosmetics are widely adulterated with phthalates such as DEP and DEHP [118].

Fragrances usually added as additives, representing a complex mixture of a dozens of chemicals are also usually adulterated. These include a broad range of compounds that can be divided in to four main groups according to their chemical structure: nitro, polycyclic, macrocyclic, and alicyclic musk fragrances [119]. Nitromusks dominated the market for many years but declined significantly in the 90s due to their bio accumulative properties and adverse health effects [120]. They are easily absorbed by organic matter and accumulate in the sediments making them a potential contamination source [121]. This is a threat to aquatic ecosystems and food safety. In this respect, musk tibetene, musk moskene and musk ambrette are prohibited in terms of the European Directives 98/62/EEC and 1223/2009/EEC [122]. Recently, the European Commission under the new chemical regulation Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), considered musk xylene a very persistent and bio accumulative substance, so they decided to prohibit it as well [123].

To ensure microbiological quality, consumer safety and the organoleptic properties of cosmetic products, manufacturers use preservatives [124]. They inhibit the growth of microorganisms in cosmetics (antimicrobial function) but may also be added to protect such products against damage and degradation caused by the exposure to oxygen (antioxidant function) [125]. Some preservatives such as parabens are known to modulate and disrupt the endocrine system [126]. There is also current scientific evidence that indicates that the use or misuse of biocidal products may contribute to the increased occurrence of antibiotic-resistant bacteria, both in humans and in the environment [127]. For this reason, some forms of parabens are prohibited in Denmark (propyl and butyl paraben, their isoforms, and salts) in cosmetic products for children up to 3 years of age [128]. The European Directive 1976/L0768 relating to cosmetic products and its successive amendments permit the use of parabens at a maximum concentration of 0.4% (w/w) each and a total maximum concentration of 0.8%

(w/w), expressed as p-hydroxybenzoic acid equivalent [129]. Additionally, some heavy metals such as mercury are incorporated as preservatives in cosmetic products because they possess antibacterial and antifungal properties [130].

Of all the cosmetic adulterant mentioned above, heavy metals are the most common. Heavy metals such as lead, mercury, cadmium, arsenic, and nickel, are also found in various types of cosmetics as pigments, preservatives, UV filters as well as antiperspirants, antifungal, and antibacterial agents [130, 131]. Some metallic compounds are routinely used in cosmetics as they possess properties to peel and lighten the skin [132]. In the manufacturing of colored cosmetics, heavy metals such as cobalt, nickel, aluminum and iron may be added as pigments [131]. Cobalt and nickel are commonly found in lipstick, eyeshadow, face paint and hair cream. Cobalt and its salts are widely used as coloring agents in makeup and light-brown hair dyes. Iron oxides are common colorants in eye shadow, blushes, and concealers [133]. Some aluminum compounds are colorants in lip glosses, lipsticks, and nail polishes [134].

In cosmetic products, organic mercury compounds, such as thiomersal, methylmercury, and ethyl mercury, are used as preservatives [4]. Additionally, mercury salts, notably mercuric amido chloride or ammoniated mercury, mercuric chloride, and mercurous chloride or calomel, are forms of mercury commonly added to skin lightening products [5]. Mercury salts repress the production of melanin in dermal cells. In the production of melanin, Tyrosinase, catalyzes the hydroxylation of the amino acid L-tyrosinase to 3,4-dihydroxyphenylalanine (DOPA) and the conversion of DOPA to dopaquinone. Mercurous and mercuric ions inhibit melanin production by competitive inhibition of copper in tyrosinase and subsequent inactivation of tyrosinase. These ions bind to the catalytic center of the enzyme and the inhibition cannot be reversed [102]. The onset and severity of mercury poisoning health effects depend of various exposure factors, such as the concentration of mercury in the product, compounds in the product that affect mercury solubility, one's skin characteristics, time length of exposure and others, all affecting the distribution of mercury in the body [135]. For this reason mercury poisoning is challenging to diagnose. The symptoms of short term mercury expo-

sure include, coughing, shortness of breath, nausea and sore throat. However if exposed to mercury for a longer time, one may suffer from tremors, loss of appetite, anxiety, and variation in sight or auditory perception [103]. If ingested, mercury may cause indigestion. It may also inhibit xanthine oxidase function [136]. Dermal exposure to mercury compounds can cause dermatitis, irritation, and allergies [137]. Once in the blood stream, mercury accumulates in various organs resulting in decreased function [138].

The world's leading health regulatory institutions restrict the use of mercury in cosmetics. The EU Cosmetics Regulation (EC) 1223/2009, prohibits mercury containing cosmetic products in the EU market except for the use of thiomersal in eye products where a maximum concentration of 70 ppm of mercury is permitted in eye makeup, mascara, and cleansing products [12]. The US Food and Drug Administration (FDA) under title 21 CFR 700.13, limits the amount of mercury in cosmetic products to a trace amount (1 ppm) unavoidable under good manufacturing practice except in those products intended for use around the eye, which it limits to 65 ppm [13]. Despite strict regulation and monitoring, consumers continue to have access to mercury based cosmetic products and sporadic cases as well as outbreaks of mercury poisoning still occur [4, 139, 140]. Due to the mentioned health hazards to consumers and the resulting economic losses of adulteration, it is necessary to employ highly sensitive, accurate, timeous, and selective analytical methods for monitoring, surveillance as well as analysis of adulterants.

2.3 Analysis of Adulterants in Food and Cosmetics

Food analysis should expand beyond improving nutritional profile, transparency of ingredients and regulation of unhealthy foods to regular monitoring, surveillance and adulterant detection in food products to ensure public well-being, prevention of food borne illnesses and reduction of adulterating cases [141]. Similarly, as a paramount priority, cosmetic products placed on the market must be safe to consumers and shall not cause any damage to human health under customary or reasonably foreseeable conditions of use [90]. Therefore, the need to guarantee product safety and enforce regulatory compliance in both food

and cosmetics, calls for the development of effective and convenient methods for analysis of illegal ingredients, with high molecular specificity and sensitivity. To date, a plethora of analytical instruments have been reported for the analysis of adulterants in food and cosmetics, especially thiocyanate in milk and mercury in cosmetics.

Thiocyanate has been analyzed using sophisticated, sensitive instrumentation, mainly based on electrochemical, spectroscopic and chromatographic techniques. Historically, the first analytical approaches for the determination of thiocyanate in biological fluids were based on spectrophotometry [142–147], potentiometry with ion-selective electrodes [148, 149], and fluorimetry [150, 151]. However, the most frequently employed methods were based on the spectrophotometric detection of coloured complexes; for example, Bowler's method exploited the reactivity of SCN^- with Fe (III) in acidic media to yield a reddish complex $[\text{Fe}(\text{SCN})_3]$ [152, 153]. However the coloured complex formed was unstable, and the method was not selective [153].

Recently UV-visible spectrophotometry techniques based on ion-pair formation, oxidation (reaction with 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol) or reduction reactions have been used in the analysis of SCN^- [142]. Fluorescence techniques based on modified König method have also been reported [154, 155]. Though some of these presented high sensitivity, they were subject to interferences caused by concomitant species [148, 149, 151, 156]. Others were fast and simple but presented low sensitivity [142, 157]. Furthermore, some proposed spectrophotometric methods required complex instruments [158, 159], or the use of hazardous reagents [156].

Chromatographic techniques that have been reported for the chemical analysis of thiocyanate including, capillary electrophoresis (CE) [26, 27], ion chromatography [20–24], gas chromatography (GC) with flame ionization detection [34], electron capture detector [32, 33] or coupled with various types of mass spectrometers [35], high-performance liquid chromatography (HPLC) using ultraviolet [28], electrochemical detection [29] or coupled with various types of mass spectrometers [30, 31]. The CE technique offers the potential for the development of methods for complex samples with fewer sample preparation steps and short elution

times, using low reagents and samples quantities. In this technique, fused silica capillaries are frequently used, where the electroosmotic flow (EOF) is generated due to the ionization of silanol groups. Several parameters affect EOF stability [25]. Any variation in the EOF parameters changes the migration time of analytes [160]. Milk is a complex matrix, it contains large quantities of proteins that can be adsorbed onto the inner wall of a fused silica capillary, due to different hydrophobic and electrostatic interactions. This usually causes challenges, such as the instability of EOF and poor repeatability [161].

The detection of SCN^- in complex matrices such as milk using DAD [20], conductivity [21, 22], MS either in single state [23] or tandem [24] after IC separation has also been reported. Although these methods presented high efficiency some of them are subject to interferences caused by other analytes. Only a few methods were designed for the detection of SCN^- alone, most ion chromatography methods simultaneously detect SCN^- , perchlorate, iodine, and nitrates [9]. Therefore, stable labelled isotopes of thiocyanate must be added as an internal standard that either enhance or suppress SCN^- signal to compensate for matrix effects as well as correct for recovery [20].

After GC separation, SCN^- has been detected using an electron capture detector [32, 33], a flame ionization detector (FID) or a flame thermionic detector (FTD) [34] and mass spectrometry [35]. Although these detectors have advantages and disadvantages, MS detectors are preferred, due to their extreme sensitivity and the ability to perform stable isotope dilution. However, thiocyanate is not volatile [35]. It is usually derivatized to a semi-volatile compound for GC analysis [162]. The analysis of SCN^- with HPLC also requires derivatization. HPLC determination of SCN^- as a pentafluorobenzyl derivative has been reported [163], but the resulting derivative is not sensitive enough by ultraviolet detection for the trace analysis of SCN^- . A sensitive and specific HPLC method with fluorometric detection has been described for the trace determination of SCN^- [164]. However, the method also involved the derivatization of SCN^- with 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one (Br-MBX) hence an extra step was added to the analysis.

Likewise, a variety of techniques have been used for the determination of trace mercury in a

wide spectrum of samples. They include spectrometric techniques (atomic absorption spectrometry (AAS), atomic fluorescence spectrometry (AFS), inductively coupled plasma optical emission spectrometry (ICP-OES), inductively coupled plasma mass spectrometry (ICP-MS), spectrophotometry and spectrofluorometry, and electrochemical techniques (voltammetry and potentiometry) [165]. AAS methods fall into two categories: flame (FAAS) and flameless. Flameless methods are cold vapor AAS (CVAAS), intended specifically for the analysis of mercury, and hydride generation AAS (HGAAS) [166]. The determination of mercury by hydride generation AAS [36], and cold vapor AAS [37] has been reported. Compared to AAS and AFS, ICP-MS offers much better sensitivity with capabilities for multi element and isotope ratio measurement [167]. Furthermore, ICP-MS has a good detection limit, unfortunately the use of argon limits its use [168]. Also, ICP-MS often has a pronounced memory effect from mercury. This is due to retention of mercury in multiple locations of the ICP-MS system such as the sample introduction tubes, the nebulizer and the spray chamber, as well as the torch [169]. Additionally, in ICP, the ionization efficiencies for hard-to-ionize elements such as mercury are considerably low resulting in relatively poor analytical sensitivity for these elements [167].

Chemical vapor generation (CVG) has been used to enhance sensitivity in the detection of mercury. The technique was originally developed as an introductory technique for AAS, although it was later used for other techniques, most notably AFS [170]. Atomic emission spectrometry (AES) and ICP-MS coupled with CVG separation have also been applied in the determination of mercury [167]. Among these, cold vapor atomic absorption spectrometry (CV-AAS) as well as cold vapor atomic fluorescence spectrometry (CV-AFS) have received great attention [171]. However, interferences are also a common challenge when conducting analyses by CVAAS [169]. As already mentioned, mercury exists in cosmetic products in the form of organic mercury compounds and mercury salts [5]. Therefore, the speciation of mercury compounds is important due to the varying levels of toxicity associated with the different forms that Hg can take [170]. In Hg speciation analysis, different chromatographic techniques can be used for the purpose of separation. The most used chromatographic techniques are LC, GC, CE, and IC [172]. The main advantage of using HPLC separation in Hg

speciation analysis is its compatibility with various detectors like ICP-MS [173–176], CV-AFS [177–179], HG-AFS [180], DAD [181, 182], CV-AAS [182] and others like MS, AES, and UV. In addition, the most laborious, time and chemical consuming derivatization step is not necessary in HPLC separation unlike in GC [172]. Although HPLC analysis of mercury does not require derivatization of the analyte, complicated sample pre-treatment steps have been reported [183, 184].

Although, these conventional methods of analysis generally offer the sensitivity required for trace contaminant analysis, they are however restricted by costly, sophisticated instrumentation that require professional personnel and long analysis times [46]. The current demand for adulterant contamination analysis requires affordable, rapid, and easy to use analysis methods that can be applied on-site at the country's points of entry and laboratories within the country, for countries that heavily relies on importation like ours, Botswana. An example of such materials are the nanomaterials. Nanomaterials possess several unique physical and chemical characteristics that make them favourable analytical tools for the on-site detection of adulterants. Along with the large surface-to-volume ratio that favors miniaturization, nanomaterials possess unique optical properties which impart them with different colors depending on their chemical environment. They can also be fashioned with a wide range of small organic ligands and large biomacromolecules. Based these capabilities, we took advantage of nanomaterials to design novel adulterant detection systems that offer significant advantages in terms of sensitivity, selectivity, reliability and practicality, for the detection of some of the most widely adulterated consumer products; mercury in cosmetics and thiocyanate in milk.

2.3.1 Nanomaterials for the Detection of Mercury and Thiocyanate

In the last few years, nanotechnology-based techniques have been successfully used for the analysis of mercury and thiocyanate. Recently, colorimetric, fluorescence, and surface-enhanced Raman scattering (SERS) approaches employing nanomaterials, have been developed for SCN^- detection [9]. With the advantage of on-site analysis, visualization, simplic-

ity, sensitivity, and no requirement for skilful technicians, colorimetric sensors have attracted more attention [185]. Metal nanoparticles (NPs) have attracted considerable attention in the colorimetric detection of SCN^- . This is due to the strong interaction of SCN^- and metal NPs [186]. Previously, SCN^- has been reported to absorb onto the surface of many metal nanoparticles (gold, silver, copper, and palladium) NPs and metal (gold and platinum) electrodes forming Au-S, Pd-S, Ag-S and Cu-S bonds [187, 188]. Based on Sundaram E. et al. 's literature survey on SCN^- detection, most of the reported sensors have utilized mostly Au/Ag nanoparticles and Fe (III) ions directly [189].

Due to their unique optical properties including high extinction coefficient and adjustable plasmon band, gold nanoparticles (AuNPs) have attracted fast-growing interest because analyte recognition can be directly monitored by the naked eye with high sensitivity, simplicity, and low cost, without the requirement of the sophisticated instruments [190]. In such assays, the presence of the analyte triggers the aggregation or redispersion of AuNPs which results in a colour change [191]. Aggregation of AuNPs results in a colour change of the colloidal solution because of plasmon coupling depending on the size, shape, and inter-particle distance of the AuNPs, as well as their dielectric properties and those of their local environment. Recently, tween 20-stabilized gold nanoparticles-based sensor based on aggregation mechanism was developed for SCN^- ions detection [188]. However, the developed sensor could not detect SCN^- ions in the actual clinical range and could not be employed to directly detect SCN^- ions in biological samples. Some of these AuNPs-based colorimetric probes for the detection of SCN^- exhibit relatively poor selectivity due to interferences by common anions, such as SO_3^{2-} , S^{2-} , I^- and $\text{S}_2\text{O}_3^{2-}$ [186]. In addition, AuNPs aggregation is sensitive to many external factors, this may lead to false positive signals and inaccuracies.

Compared to many sensors based on the aggregation of nanoparticles, several colorimetric sensors have been proposed based on their anti-aggregation or re-dispersion, aiming at improving the selectivity and lowering false positive signals [192]. Recently, some colorimetric methods for SCN^- detection employing anti-aggregation of AuNPs have been developed. In AuNPs anti-aggregation systems, adding an aggregation agent initially induces the ag-

gregation of AuNPs by changing their inter-particle distance [193]. The target analyte then deactivates the aggregation agent due to its higher binding affinity for the nanoparticles, preventing the aggregation of AuNPs. SCN^- can shield Au NPs and lessen the extent of aggregation due to N,N-dimethyl-1-naphthylamine [194] or cetyltrimethyl ammonium bromide (CTAB) [9]. SCN^- can also suppress the aggregation of citrate-capped Au NPs in the presence of sulfuric acid [191]. In these assays, the AuNPs surface is usually modified with specific binding ligand (such as DNA), which complicates the synthesis method and increases the corresponding cost.

In SERS, the Raman scattering intensity of the probe molecule adsorbing on nano-textured metallic surfaces can be enhanced by more than a billion times and the sensitivity can even reach the level of single molecule detection [195]. Silver colloids are said to have better SERS activity than gold and copper colloids, thus it is an excellent substrate for SERS detection [196]. Silver aggregate nanoparticles prepared according to Lee and Meise's method, induced by trichloroacetic acid (TCA) with a large SERS enhancement factor were applied to the detection of SCN^- in milk samples. However, the reported sensor was found to be unstable [89]. Droplet SERS microfluidic chip has also been developed for SCN^- ions detection using Au/Ag core shell nanoparticles [197]. Fluorometric methods relying on fluorescence resonance energy transfer (FRET), have become a leading topic in the field of fluorescent biosensors [198]. the strong quenching ability of AuNPs, FRET-based fluorescence sensors employing the strong quenching ability of AuNPs, have been developed for detecting SCN^- [199, 200].

Currently, numerous approaches based on electrochemical and optical sensors [201–203], organic chromophores or fluorophores [204–209], conjugated polymers [210], oligonucleotides [211–213], genetically engineered bacteria [214, 215], DNAase [216, 217] and proteins [218–220], as well as inorganic nanostructures, have been established for the sensitive and selective detection of Hg^{2+} ions. Unfortunately, most of these methods suffer from poor sensitivity and selectivity, low water solubility, complex synthesis procedures, and time-consuming DNA probe preparation [221]. As a convenient detection method for Hg^{2+} , colorimetric-

sensing tools have been widely reported, especially those based on the aggregation or anti-aggregation of noble metal nanoparticles [218, 222–224]. This is due to the strong metal-lipophilic interactions between Hg^{2+} and some other metallic atoms, such as gold and silver [225]. Plasmonic AuNPs have been widely exploited for the colorimetric detection of Hg^{2+} through T- Hg^{2+} -T co-ordination chemistry [212, 226, 227], and interactions with molecules such as cysteamine [228], tween-20 [229], papain [230] and thiocetic acid [231]. However, most of these assays are insensitive or have a complicated method for sample preparation. These shortcomings limit the practical application of such sensors in real samples [230]. Recently, colorimetric methods for the detection of Hg^{2+} ions relying on enzymatic catalysis have been successfully proposed [232]. However, these enzymes have a few disadvantages. For instance, their catalytic activity is sensitive to environmental conditions such as high pH, temperature, and ionic strength [233]. Faced with such obstacles, artificial enzyme mimics, known as nanozymes have emerged as a robust alternative to efficiently avoid the above mentioned drawbacks of natural enzymes.

2.3.2 Nanozymes for the Detection of Mercury and Thiocyanate

Nanozymes are inorganic nanostructures with intrinsic enzymatic activities. Inspired by nature but advantageous over natural enzymes, nanozymes have facile and low-cost synthesis, they have high stability under stringent conditions and can be produced to give high yield [234]. Moreover, nanozymes have adjustable activity, and diverse functions, making them potential substitutes and valuable competitors for natural enzymes [235]. Nanozymes may be classified based on their catalytic-like activity or their composition.

2.3.2.1 Classification Based on Catalytic-like Activity

Gao et al. [236] were the first to learn that nanoparticles (NPs) could exhibit intrinsic peroxidase (POD)-like activity. Subsequently, other nanomaterials that exhibited POD-, catalase (CAT)-, superoxide dismutase (SOD)-, or oxidase (OXD)-like catalytic activities, were discovered. Recently, several colorimetric sensors based on nanozymes for detection Hg^{2+}

[45, 47, 50–52, 221, 225, 232, 233, 237–239] and SCN^- ions [186, 240–243] have attracted attention due to their distinct variation in colour. They possess intrinsic biological enzyme-like catalytic activities, predominantly, catalytic activities like peroxidase [45, 47, 50, 51, 186, 221, 225, 233, 238, 240–242, 244] oxidase [244–246] and catalase [233, 238, 247] like activities.

Peroxidase-mimics catalyze the oxidation of chromogenic substrates in the presence of hydrogen peroxide (H_2O_2). In such reactions, H_2O_2 adsorbs onto the surface of the nanozyme and breaks into hydroxyl radicals ($\bullet\text{OH}$). Chromophore substrates that include benzidine and its derivatives, such as 3, 3'-diaminobenzidine (DAB), 3,3',5,5'-tetramethylbenzidine (TMB), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), o-phenylenediamine (OPD) and N,N-dimethylphenylenediamine (DMPD) oxidize in the presence of $\bullet\text{OH}$ radicals and produce a colour change [248]. Many colorimetric assays for the detection of mercury and thiocyanate were developed using the chromogenic oxidation of these substrates. TMB is the more popular substrate due to its nontoxicity [249]. The oxidation product of these substrates is a coloured cation radical that is used as the analytical probe [250]. The intensity of the colour is proportionally related to the concentration of the target analyte [251]. Mercury and thiocyanate have been reported to either enhance or suppress the peroxidase-like activity of nanozymes, a characteristic we took advantage of in these thesis.

To the best of our knowledge only five nanozymes, all exhibiting peroxidase-like activity have ever been synthesized for the detection of SCN^- . In two of these, Ahmed et. al. [241, 242] synthesized gold nanoparticle-graphene quantum dots (GQDs-Au NPs) hybrids and positively charged gold quantum dots (Au QDs) respectively, each exhibited peroxidase-like activity. In both cases Ahmed et. al. found out that SCN^- exhibited hydroxyl radical ($\bullet\text{OH}$) scavenging activity. This effect boosted the nanozymatic activity of GQDs-Au NPs and Au QDs to detect SCN^- . The third nanozyme developed by He et. al. [240] involved coral-like bimetallic PdCu nanostructures with enhanced peroxidase-mimicking properties. Interestingly, they found out that SCN^- inhibited the catalytic activity of PdCu selectively. They further discovered that, SCN^- adsorbed onto the surface of PdCu via the binding interaction of SCN^- and the Pd atoms. The adsorption decreased the active sites exposed on the

PdCu peroxidase mimic, and that resulted in the inhibition of the catalytic reaction. In the forth, core-shell Au@Pt nanocatalysts (Au@Pt NCs) with high peroxidase-like activity were synthesized [186]. Similarly, in the presence of SCN^- , the catalytic activity of Au@Pt NCs decreased. The decrease in catalytic activity of Au@Pt NCs was attributed to the binding of SCN^- and the Pt species on the surface of Au@Pt NCs which suppressed the Pt activity. Alula and Feke have recently reported a fifth nanozyme-based SCN^- sensor in which silver nanoparticles coated filter paper (AgNPs/FP) exhibited peroxidase-like catalytic activity. In their work, thiocyanate also exhibited catalytic inhibition property by blocking the active sites of the enzyme mimetic through sulphur-silver bond formation [243].

For Hg^{2+} detection, numerous colorimetric sensors have been developed based on the enhanced peroxidase-like activity of the nanozymes by Hg^{2+} with TMB as a substrate [47, 50, 225, 239, 244, 252–254]. On the other hand, nanozymes based on Hg^{2+} induced inhibition of peroxidase-like activity have also been developed. For instance, Zhou and Ma [255] proposed a one-step method that simultaneously applied fluorescent and colorimetric dual-readout for Hg^{2+} detection, based on the inhibited peroxidase-like activity of Pt nanoparticles (Pt NPs) for the oxidative reaction of a fluorogenic substrate o-phenylenediamine (OPD) with H_2O_2 .

Contrary to nanozymes with peroxidase-like activity, nanozymes with oxidase-like activity can oxidize their substrate in the presence of molecular oxygen and the catalytic reaction of oxidase-like nanozymes does not require H_2O_2 . Instead, they generate H_2O_2 and in few cases superoxide radicals [256]. As already stated, all the nanozymes that have been developed for the detection of thiocyanate employed the peroxidase-like activities of those nanozymes. However, in our work, for the first time ever we employed bio-synthesized AgNPs with oxidase-like activity for the detection of thiocyanate. This approach uses oxygen as a green oxidant instead of H_2O_2 which is destructive and unstable. Nanozymes with enhanced oxidase-like activity in the presence of mercury have been synthesized [245, 246, 257]. For example, Liu et. al. [246] constructed a nanozymatic Au-NiFe LDH/rGO nanocomposite with enhanced oxidase-like activity in the presence of mercury. They found out that the en-

hanced catalytic-like activity was due to the increased total radicals including $O_2^{\bullet -}$ from the electron capture by oxygen molecules during the oxidase-like catalytic reaction as a result of the generation of Hg^0 to form Au-Hg and $\bullet CH_3$ from the degradation of MeHg. In another study, a fluorescence assay for the detection of Hg^{2+} using a gold nanoparticle (AuNP)-based probe was proposed by Yan et. al. [257]. The assay was based on the formation of Hg-Au alloys, which accelerated the oxidization of o-phenylenediamine by dissolved oxygen to produce 2,3-diaminophenazine, a fluorescent product. Similarly, mercury (II)-stimulated oxidase mimetic activity of citrate-capped silver nanoparticles (Cit-AgNPs) have been studied [245]. An Hg-Ag alloy with high catalytic activity towards the oxidation of TMB, in the presence of dissolved oxygen was formed due to the reduction of Hg^{2+} on the Cit-AgNPs surface. In contrast to the idea that the Hg^{2+} could enhance the oxidase-like catalytic activity of nanozymes, some nanozymes with inhibited oxidase mimetic activity towards TMB in the presence of Hg^{2+} have been constructed [52, 258]. For instance, Wang et. al. [52] fabricated graphitic carbon nitride supported platinum nanocomposites (g-C₃N₄/PtNPs) with inhibited oxidase-like activity in the presence of Hg^{2+} , that was brought about by the formation of a Hg-Pt amalgam. Guo et.al. [258] also fabricated platinum(Pt)-selenium(Se) nanostructures with inhibited oxidase-like activity in the presence of Hg^{2+} for the detection of Hg^{2+} due to the metallophilic interactions between Hg and Pt-Se nanostructures.

Nanozymes with catalase-like activity decompose H_2O_2 into water and oxygen. Fewer nanozymes with catalase-like activities have been fabricated for the detection of Hg^{2+} [233, 238, 247]. In the recent past, there have been studies characterized by a switch from peroxidase-like activity to catalase like activity of nanozymes in the presence of Hg^{2+} . For instance, Tseng et.al. [238] synthesized bimetallic platinum/gold nanoparticles (Pt/Au NPs) which were found to exhibit peroxidase-like activity, and the deposition of mercury was found to switch the enzymatic activity to a catalase-like activity. The peroxidase catalytic activity of Pt/Au NPs was switched to catalase-like activity in the presence of mercury ions due to the strong metallic bond that exists between Au and Hg. Similarly, Lien et. al. [233] have developed a Bi^{3+} -Au NPs nanozyme with peroxidase-like activity towards the H_2O_2 -mediated oxidation of Amplex Red (AR), for the selective detection of Hg^{2+} and in the presence of

mercury its peroxidase-like activity was also switched to catalase-like activity. In another study, most recently, Wang et. al. fabricated gold-palladium modified zirconium metallorganic frameworks (AuPd@UiO-67) nanozyme with catalase-like activity as a signal enhancer for sensitive detection of Hg^{2+} [247]. Here, a current signal was created by means of the catalytic action of AuPd@UiO-67 nanozyme toward H_2O_2 . When the concentration of Hg^{2+} was raised, the quantity of AuPd@UiO-67 nanozyme modified on the electrode increased, thereby realizing the detection of Hg^{2+} .

In another class of nanozymes, reductase-mimics catalyse the sodium borohydride-induced reduction of dyes such as 4-nitrophenol, methyl orange and methylene blue to colourless products [259]. The reduction of methylene blue and 4-nitrophenol using Au-based reductase-like nanozymes as catalysts for the colorimetric detection of Hg^{2+} has so far been reported [45, 221, 232]. For example, in a study by Chen et. al. [45] citrate capped AuNP were used to catalyse the reduction of 4-nitrophenol by NaBH_4 . The Hg^{2+} ions adsorbed on the Au NPs surface to form a gold amalgam, which initiated the catalytic activity of Au NPs to catalyse the dye reduction. Similarly, Wang et. al. [232] synthesised Au NP anchored Fe-TCPP-MOF and Hai et al. [221] encapsulated Au NP in porous wood as reductase mimics for the colorimetric detection of Hg^{2+} . In both cases methylene blue was used as the signalling colour reporter. For our work, we synthesized a nanozyme belonging to this class of nanozymes, reductase mimics. We fabricated Au@ZnO/ Fe_3O_4 hybrid material with reductase-like catalytic activity towards the reduction of 4-nitrophenol in the presence NaBH_4 with inhibited catalytic activity in the presence of Hg^{2+} for the detection of Hg^{2+} .

2.3.2.2 Classification Based on Composition

Since the first report by Gao et. al. that Fe_3O_4 exhibits intrinsic peroxidase-like activity inorganic nanozymes of varying composition mainly metal-based nanozymes (e.g. gold [45], platinum [255], palladium [240] and silver [245]) carbon-based nanozymes, (e.g. graphene [52, 247]), metal organic frameworks [232] and their combined nanostructures (hybrids) [50, 241, 258], have demonstrated their ability to mimic enzymatic activities with reports on their

potential applications in the detection of Hg^{2+} and SCN^- . Most nanozymes for the detection of Hg^{2+} and SCN^- are Au-based. As already stated above, of the five nanozymes reported for SCN^- detection three are Au-based - [242], gold nanoparticle-graphene quantum dots [241], and Au@Pt nanocatalyst [186], one has a coral-like bimetallic PdCu structure [240] and the other is Ag-based [243]. As for the Au-based nanozymes for the detection of Hg^{2+} , some are citrate reduced AuNP [45, 239], others are hybrids of Au with other metals [233, 254], DNA [226, 253], polymers [51, 257], MOFs as supporting material [232, 247], and other interesting supporting materials such as wood [221] and paper [252]. A few non-Au based nanozymes for the detection of Hg^{2+} have also been reported [52, 244, 245, 258].

It is well-known that Au surface exhibits a strong affinity for Hg^{2+} [45]. Thus, it is not surprising that most nanozymes for the detection of Hg^{2+} are Au-based. Au selectively interacts with Hg^{2+} to form an amalgam. This Au-Hg alloy usually exhibits an enhanced catalytic-like activity compared to Au alone. In their most basic form, citrate capped AuNP have been synthesized for the detection of Hg^{2+} [45, 239]. Gold based hybrid materials, in which the components bring distinct properties and the combination have offered new and enhanced properties have been developed. Citrate reduced AuNP functionalized with DNA, have shown improved catalytic-like activity for the detection of mercury [213, 225].

Additionally, some bimetallic Au-based NP have been reported to have mercury-induced switching of enzyme-like activity. For example, Bi^{3+} -AuNPs were observed to switch from peroxidase-like to catalase-like nanozyme after reaction with Hg^{2+} [233] and in similar work, Pt/Au NPs were found to exhibit peroxidase-like activity, which was switched to catalase-like activity after deposition of mercury [238]. Moreover, Pt and Pd have been proven to be better catalase-mimics than Au [260]. Hence, Au based nanozymes for the detection of Hg^{2+} based on their catalase-like activity have been modified with either Pt or Pd [238, 247]. MOFs have also been used as supporting matrix for immobilization of the AuNP-based nanozymes [232, 247]. This has been reported to prevent aggregation thus leading to higher catalysis properties of the formed mercury amalgam [232].

Non-Au based nanozymes for the detection of Hg^{2+} have also been reported. Platinum NPs

(PtNPs) are said to form an amalgam with mercury [255]. In one scenario citrate reduced PtNPs showed enhanced peroxidase-like activity [255] and in others, graphitic carbon nitride supported platinum nanocomposites [52] and a bimetallic Pt-Se nanozyme [258] have been reported to exhibit suppressed-oxidase like activity in the presence of Hg^{2+} . In contrast Hg^{2+} simulated oxidase mimetic activity of silver nanoparticles has been reported [245].

2.3.2.3 Physical and Kinetic Characterization of Nanozymes

The size and shape of nanozymes are key variables that affect their catalytic-like activity. This kind of effect is inherently related to various surface chemistries of nanozymes [261]. Therefore, to fully comprehend and predict the catalytic behaviour of nanozymes, characterization is a crucial step. Several physical characterization techniques have been devised, the most common ones include, energy dispersive X-ray spectroscopy (EDX), dynamic light scattering (DLS), X-ray photoelectron spectroscopy (XPS), UV-Vis spectroscopy, fluorescence spectroscopy, powder X-ray diffraction (PXRD), scanning electron microscopy (SEM), atomic force microscopy (AFM), fourier transform infrared spectroscopy (FTIR) and transmission electron microscopy (TEM) [262]. Each technique provides useful insight into one or more of the size, shape, size distribution, stability, surface functionalization, composition, morphology, and activity of nanozymes [263]. In most cases, nanozymes are characterized employing several related techniques in concert, to remove ambiguities associated with the use of a single technique, and to have a clear understanding of the material properties.

Nanozyme size, shape and morphology can be determined at the single-particle level at sub-nanometer resolution using high resolution microscopy techniques, such as SEM, TEM and AFM providing accurate details on the size, shape and texture of the nanoparticles being studied. These characterization techniques are based on the interaction between the atomic structure and the impinging electron beam, or with a scanning probe [262]. In most cases, in the imaging of nanozymes for the detection of Hg^{2+} and SCN^- , TEM was used. For instance, Tseng et. al. [238] assessed the effect of varying the molar ratio of Pt/Au on the size and hence catalytic activity of the nanozymes using TEM. They found that, as the

$[\text{Pt}^{4+}]/[\text{Au}^{3+}]$ molar ratio increased, the sizes of the Pt/Au NPs decreased, primarily because of the higher seeding rate in the presence of Pt^{4+} . When studying the morphology of a Pt/Se bimetallic nanozyme they had constructed of the detection of Hg^{2+} , Guo et. al. [258] found that Pt nanoparticles possessed spherical-like geometry however after modification, the Pt-Se nanostructures had an irregular shape. In contrast Hai et. al. [221] found that AuNP had no bearing on the 3D porous structure of the wood they had used to encapsulate the AuNP. In addition, DLS has been used to study effect of the Hg^{2+} and SCN^- on the size of the nanoparticles by measuring the hydration kinetic particle size of nanozymes before and after incubation with the analyte. Based on Peng et al. 's work, DLS measurements revealed that the hydration average size of Au@Pt NCs increased from 27.9 nm to 88.8 nm after modification with cystine but decreased to 63.5 nm in the presence of SCN^- [186]. Furthermore, Jiang et. al. reported that, their DLS measurements showed that the average hydrodynamic diameters of the AuNPs increased from 39 to 126 nm after incubation with Hg^{2+} [51].

The selective synthesis of nanozymes with varying specific crystal planes is an important prerequisite for exploring their shape-dependent catalytic activity employing XRD and TEM. The effect of nanozymes' shape on their catalytic activity is complicated and this can be appreciated from varying view points. On one hand, the varying structures among the different shapes distinctly create a variation in crystallographic surfaces. On the other hand, the surface physicochemical properties of these nanomaterials is highly dependent on the coordination environment and number of dangling bonds of different crystal plane atoms, which affect the catalytic activity. XRD employs X-rays to obtain the diffraction patterns of nanozymes. XRD diffraction patterns provide details on the composition and crystalline phase of nanozymes by analysing and comparing distinctive diffraction patterns with standard patterns in the database. For instance, Huang et. al. [244] prepared CS-MoSe₂ NS, by simultaneous exfoliation and functionalization via a one-step ionic liquid-assisted grinding method. The crystal structure of the as-exfoliated CSMoSe₂ NS was measured by XRD. They found that the diffraction peaks of bulk MoSe₂ and the as-exfoliated MoSe₂ nanosheets were well matched to a database known as JCPDS Card 17-0887. Furthermore, to confirm efficient

exfoliation of MoSe_2 they studied the diffraction patterns of their nanozyme after exfoliation, they found that some of the peaks still existed while the others disappeared, indicating the efficient exfoliation of MoSe_2 . Zhang et.al. [50] also characterized the crystal structures of their $\text{Au/Fe}_3\text{O}_4/\text{GO}$ hybrid by powder X-ray diffraction (XRD) analysis. From their results their diffraction patterns matched well with those of standard GO, Fe_3O_4 (JCPDS, No. 19-629) and Au (JCPDS, No. 89-3697) powder diffraction data indicating the successful synthesis of $\text{Au/Fe}_3\text{O}_4/\text{GO}$ hybrid.

In other studies, He et. al. [240] and Guo et. al. [258] each synthesized bimetallic nanozymes which were characterized by XRD. In the XRD studies of their synthesized bimetallic nanozymes, He et. al. conducted XRD measurements to differentiate the PdCu from the pure Pd. Likewise, Gou et. al. collected XRD patterns of Pt and Pt-Se nanozymes they had synthesized. He et. al. found out that the only observable peaks on the pattern belonged to PdCu and no other peak, such as metallic Cu and its oxides, was present. Similarly, Gou et. al. found that in the Pt-Se nanostructures pattern, only Pt lattice could be indexed. He et. al. concluded that the absence of the Cu diffraction peaks on the PdCu diffraction pattern indicated that the Cu entity had been immersed into the Pd lattice through microwave irradiation. Whereas Guo et. al. said that Pt-Se nanostructures formed with doped Se might possess similar crystal form with pure Pt nanoparticles hence only Pt diffraction peaks were observed. In both cases, the diffraction peaks of the bimetallic nanozyme had shifted to the right compared to that of the pure metal. He et. al. attributed this to the integration of Cu atoms into the Pd lattice in place of Pd atoms and Guo et. al. said it may be due to the doped Se.

TEM has also been used to study the crystalline structure in selected microscopic regions of crystalline materials by spatially confining and focusing the impinging beam and detecting patterns from the resulting electron diffraction [263]. In addition to XRD analysis, He et. al. [240] used TEM to study the crystalline nature of their nanozyme, they found that pure Pd had long-range order lattice fringes, which was indicative of its excellent crystallinity. The measured 0.225 nm lattice spacing matched well with the (111) plane of face-centred cubic Pd. In PdCu , short-range order lattice fringes were captured suggesting that the bimetallic

PdCu still had some crystallinity in their structure. The lattice spacing of Pd decreased to about 0.218 nm for the Pd (111) facet. They ascribed the phenomenon to the intercalation of Cu atoms into the Pd lattice. As Cu has a smaller atomic size than Pd, the intercalation of Cu caused crystal defects, which led to the decrease of the Pd lattice spacing. Hai et. al. [221] had also used TEM to study the crystallinity of the Au NPs in their Au@wood nanozyme, and the measured lattice fringes correspond to a spacing of 0.24 nm, which matched well with the expected spacing of the (111) plane of the Au.

In the design and construction of hybrid nanozymes, the combination of EDX, TEM and SEM have been used to provide information about elemental composition. In addition, XPS measurements have been performed to determine the chemical states and compositions of nanozymes. This information has been used to confirm modification. For instance, after synthesizing an AuNP@MOF for detecting Hg^{2+} , Wang et.al. [232] compared TEM images of the as-synthesised MOF, the AuNP and AuNP@MOF to confirm construction of the hybrid. In another study, Zhang et.al. [50] obtained TEM images of the GO and Au/ Fe_3O_4 /GO hybrid used in the detection of Hg^{2+} . Based on the images and elemental mapping results, they concluded that Au and Fe_3O_4 NPs were anchored at a specific location of GO during the assembly, and the Au and Fe_3O_4 NPs were strongly coupled onto the GO surface. To investigate the composition of their Pt-Se nanostructures, Guo et. al. [258] collected XPS spectra of Pt 4f and Se 3d regions. The XPS spectrum of Pt 4f region in Pt-Se nanostructures revealed a Pt $4f_{7/2}$ peak at about 71.5 eV and Pt $4f_{5/2}$ peak at 74.4 eV. The XPS spectrum of Se 3d region in Pt-Se nanostructures showed that the 3d orbital binding energy of Se was about 55.1 eV. Based on these results they concluded that the Pt-Se nanocomposite consisted of Pt^0 and Se^0 . Wang et. al. [52] performed XPS measurements to analyze the chemical composition of their synthesized g- C_3N_4 /PtNPs nanocomposite. They observed Pt, C and N, and concluded that the g- C_3N_4 /PtNPs was successfully formed.

FTIR has been used to obtain the functional groups of nanozymes. For instance, Wang et. al. [52] collected an FTIR spectrum to investigate the chemical functional groups of their g- C_3N_4 nanosheet support. They assigned a peak at 808 cm^{-1} as the characteristic bending mode of

triazine units. The peaks in the 1200-1600 cm^{-1} region were assigned to the stretching vibrations of aromatic C-N heterocycles and the broad band located in the 3000-3500 cm^{-1} region was related to N-H stretching modes of amino groups, which confirmed that the nanosheets had been successfully formed. Jiang et. al. [51] on the other hand, obtained the absorption bands from the functional groups of the Chitosan-AuNPs by FTIR. They attributed bands at 1650 and 3500 cm^{-1} to the presence of amino groups and hydroxyl in the chitosan-AuNPs, respectively. That marked the presence of chitosan.

As emerging alternatives to natural enzymes, it is important to understand the catalytic activity, kinetic parameters, and mechanisms of nanozymes. These can be used to evaluate the catalytic efficiency of nanozymes and to make comparison between nanozymes and the corresponding natural enzymes. The catalytic activity and progress of catalytic reactions of nanoparticles is studied by monitoring the absorbance or fluorescence signal of a chromogenic or fluorescent substrate. Here the rate of the reaction is studied in the absence of the analyte. Thereafter an analyte is added to determine the effect of the analyte on the catalytic rate of the nanozyme. In the construction of a hybrids, the intrinsic catalytic activity of each component is studied and compared to that of the nanocomposite. The catalytic performance of nanozymes is dependent on their kinetic characterization. The steady-state kinetic measurements are carried out by recording the time-dependent absorbance or fluorescence at a time interval and from this Michaelis–Menten curves are obtained. The Michaelis–Menten equation on **equation 2.1** represents the relationship between the initial velocity of the enzymatic reaction and the substrate concentration[264];

$$V = \frac{(V_{max} \times [S])}{(K_m + [S])} \quad (2.1)$$

Where V is the reaction rate, V_{max} is the maximum reaction velocity, [S] is the concentration of the substrate and K_m is Michaelis's constant. In Michaelis–Menten equation, there are two basic parameters, K_m and V_{max} . The Michaelis–Menten constant (K_m) is a characteristic physical quantity of an enzymatic catalyst and its corresponding substrate which is the

concentration of the substrate when the enzymatic reaction reaches half of the maximum velocity, V_{max} . K_m represents the affinity between an enzyme and its substrate [265]. Low K_m values reflect high affinity of the enzyme for the substrate. V_{max} describes the reactivity of the enzyme (at a fixed concentration) when saturated with the substrate [266]. In addition to direct reading of the approximation of V_{max} and K_m from the plot of substrate concentration versus reaction velocity, it is more convenient to get their exact values from the corresponding Lineweaver–Burk plot which follows **Equation 2.2** [263];

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} \quad (2.2)$$

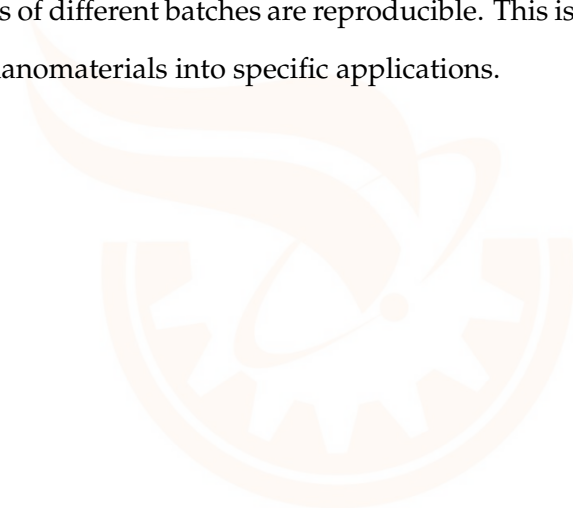
In one kinetic study, Wang et. al.[52] plotted a Michaelis-Menten curve in a defined range of TMB concentrations, in order to evaluate the enzyme mimic activity of their g-C₃N₄/PtNPs. They determined steady-state kinetic parameters from a Lineweaver-Burk plot. Furthermore, they studied steady-state kinetic assays of g-C₃N₄/PtNPs after treatment with Hg²⁺. The apparent kinetic parameters were calculated based on the Michaelis-Menten equation. After adding Hg²⁺, the apparent K_m value of g-C₃N₄/PtNPs increased while the V_{max} decreased, indicating that mercury-treated g-C₃N₄/PtNPs had lower catalytic activity and weaker affinity to TMB substrate. In another study, He et. al.[240] performed steady-state kinetics measurements to evaluate the peroxidase-like activity of their synthesised bimetallic PdCu in contrast to that of pure Pd. They observed typical Michaelis-Menten curves for both pure Pd and bimetallic PdCu. They then fitted their data to the Michaelis-Menten model to obtain the kinetics parameters, based on their results, the obtained K_m values for PdCu were a little larger than those for pure Pd. They related the larger K_m for PdCu to its larger V_{max} . To their surprise, the k_{cat} (the rate constant for the catalytic conversion of substrate into product) values for PdCu were double those of the pure Pd for both TMB and H₂O₂. The catalytic efficiency, defined by k_{cat}/K_m , was also higher for PdCu than for pure Pd. They concluded that the bimetallic PdCu is a preferred nanozyme with enhanced enzymatic activity and efficiency compared pure Pd with either TMB or H₂O₂ as the substrate.

Since the catalytic activity of nanozymes depends on surface chemistry, it is important to study surface composition of nanozymes to understand catalytic mechanisms. TEM and SEM have been used with EDX and XPS to study the catalytic mechanisms of prepared nanozymes. To understand the enhanced catalytic activity and efficiency in their bimetallic PdCu nanozyme He et. al.[240] were inspired to explore its underlying mechanism. They decided to study the surface chemistries of both Pd and PdCu using XPS. The Pd 3d XPS patterns of both nanozymes were deconvoluted to Pd⁰ and Pd²⁺. To assess the relative amount of each species on the surface, they used the ratio of the area sums. The quantity of Pd⁰ in the bimetallic PdCu was higher than that in pure Pd. They concluded that the insertion of Cu atom in to the Pd lattice increased the Pd⁰ percentage. Furthermore, based on the peroxidase mechanism of noble metal nanozymes, metallic M⁰ participates in the activation of H₂O₂ substrate to release hydroxyl radicals. Therefore, the increase of Pd ratio improves its activity and efficiency. They however did not study the mechanism behind the suppressed catalytic activity when SCN⁻ was introduced. Nonetheless, in their study, Peng et. al. [186] went a step further to investigate the surface property of their Au@Pt nanozyme after incubation with SCN⁻. They also used the ratio of the area sums to compare the proportion of Pt²⁺ and Pt⁰ on the nanozyme surface. They found that the ratio of Pt²⁺ to Pt⁰ increased after incubating with SCN⁻. They concluded that the decrease in catalytic activity of Au@Pt NCs was mainly due to the binding of SCN⁻ and the Pt species on the Au@Pt NCs surface, which shielded the Pt activity position. In addition, they believed the change of the Pt²⁺/Pt⁰ ratio was also responsible for the loss of catalytic activity to some extent, which made sense because based on their XPS pattern the quantity Pt⁰ on the surface of the nanozyme had significantly reduced after incubating with SCN⁻.

The catalytic activity of nanozymes is also believed to depend on the dispersion of nanoparticles in the presence of the analyte. Chen et. al.'s found out that the catalytic-like activity of their Au-based nanozyme towards the reduction of 4-nitrophenol was suppressed in the absence of Hg²⁺ and enhanced in the presence of a high concentration of Hg²⁺ (500 nM) as a result of aggregation [45]. This was evident from the TEM measurements they had made. In agreement, Jiang et. al. [51] hypothesized that the NH₂ of chitosan in their chitosan

functionalized AuNP interacted with Hg^{2+} and caused aggregation of AuNP and hence their nanozymes catalytic activity was enhanced. They also employed TEM measurements to prove their mechanism. In contrast, Logan et.al. [47] believed that the aggregation of AuNPs would have adverse effects on the catalytic performance, and general sensitivity of their OEG-AuNP nanozyme. Therefore, they used TEM imaging to ensure that the addition of Hg^{2+} ions did not have impact on the aggregation state of the AuNPs.

The properties of nanozymes as evidenced by the characterization techniques discussed are intimately connected to their catalytic activity. Furthermore it should be noted that characterization is the first step to ensure that synthesized nanomaterials possess desired properties and that the properties of different batches are reproducible. This is important for translating potential benefits of nanomaterials into specific applications.



Chapter 3

Methodology, Results and Discussion

3.1 Overview

In this chapter, the various experiments that were carried out, together with their results are discussed. The chapter is divided in to two main sections, with the first, section 3.2 dealing with the fabrication of a Au@ZnO/Fe₃O₄ hybrid material with reductase-like activity towards the reduction of 4-nitrophenol (4-NP) to 4-aminophenol (4-AP) by free radicals derived from sodium borohydride (NaBH₄) for the detection of Hg²⁺ based on Hg²⁺ induced suppressed reductase-like activity of the nanozyme, as well as the discussion of the results obtained. Section 3.3 deals with the synthesis of morula extract mediated silver nanoparticles (AgNPs) with intrinsic oxidase mimetic catalytic activity towards the oxidation 3,3',5,5'-Tetramethylbenzidine (TMB) in the presence of oxygen, a green oxidant and their application in the analysis of SCN⁻ as well as the discussion of the results obtained.

3.2 Fabrication of a Au@ZnO/Fe₃O₄ Nanozyme With Reductase-like Activity Towards the Reduction of 4-Nitrophenol for the Colorimetric Detection of Hg (II) Based on Hg (II) Induced Suppressed Enzyme-like Activity of the Nanozyme

3.2.1 Materials and Methods

3.2.1.1 Chemicals

Iron (II) sulphate heptahydrate (FeSO₄·7H₂O), iron (III) chloride hexahydrate (FeCl₃·6H₂O), zinc nitrate (Zn(NO₃)₂), auric acid (HAuCl₄), sodium borohydride (NaBH₄), mercury nitrate (Hg(NO₃)₂), sodium nitrate (NaNO₃), copper nitrate (Cu(NO₃)₂), calcium nitrate (Ca(NO₃)₂), aluminum nitrate (Al(NO₃)₃), cesium nitrate (Cs(NO₃)₂), lead nitrate (Pb(NO₃)₂), and cobalt nitrate (Co(NO₃)₂) were purchased from Sigma Aldrich (Johannesburg, South Africa). Silver nitrate (Ag (NO₃)₂), sodium hydroxide (NaOH), and ammonia (NH₄) were purchased from Merck (Darmstadt, Germany). Ethylene glycol (EG) was purchased from Rochelle (Johannesburg, South Africa). A skin lightening cosmetic cream was purchased from a local store (Palapye, Botswana). All reagents were of analytical grade and were used without additional purification. All analyte solutions were prepared in Millipore ultrapure double deionized water.

3.2.1.2 Instrumentation

The surface morphology of the prepared nanocomposite was studied using a high-resolution Carl Zeiss Gemini SEM500 Electron microscope supplied by Carl Zeiss (Berlin, Germany). X-ray diffraction (XRD) patterns were obtained on a D2 phaser XRD300 W powder diffractometer supplied by Bruker (Karlsruhe, Germany) for a 2θ range of 10-90 at scan rate of 0.05 degree/s using Cu Kα radiation at 40 kV and 100 mA. Raman spectra were measured by Horiba Labram Raman spectrometer equipped with 785-nm laser line as excitation source and a CCD detector purchased from Horiba Jobin-Yvon Technology (Lille, France). Thermo-fisher UV-visible absorbance spectrophotometer was used to collect UV-vis spectra using 1

cm glass cuvettes as sample cells (Waltham, MA, USA). An Axis Supra X-Ray Photoelectron Spectrometer (XPS) supplied by Kratos Analytical (Manchester, UK) was used to assess the surface chemical composition of the Au@ZnO/Fe₃O₄ and confirm the formation of an amalgam when Hg²⁺ ions encountered Au.

3.2.1.3 Preparation of Fe₃O₄ Nanospheres

The Fe₃O₄ magnetic nanoparticles were prepared based on the co-precipitation of Fe³⁺ and Fe²⁺ under aqueous ammonia as precipitating agent, with some modifications [267], as shown on **Figure 3.1**. 3.48 g FeSO₄·7H₂O and 3.38 g FeCl₃·6H₂O were dissolved in 100 mL deionized water and mixed under vigorous stirring. Aqueous ammonia was dropped slowly into the solution. It was observed that the solution became black. The black mixture was then aged at 85 °C for 2 h. After that, the product prepared was allowed to cool overnight. Finally, the black particles were washed with deionized water and dried in the oven for 6 h at 70 °C.

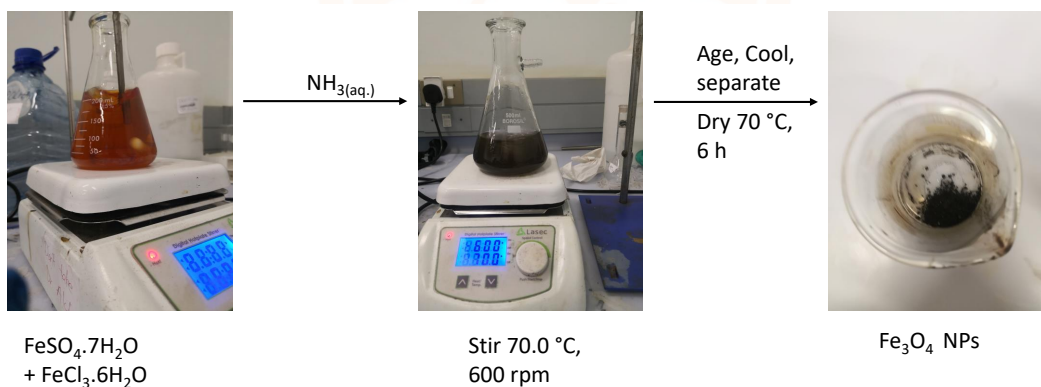


Figure 3.1: Co-precipitation Synthesis of Fe₃O₄ Nanospheres

3.2.1.4 Preparation of ZnO/Fe₃O₄ Composite

In preparing the ZnO/Fe₃O₄ composite, we employed a previously developed method with minor modification [268] as shown on **Figure 3.2**. 0.15 g of Fe₃O₄ was dispersed in 200 mL of 45 mM zinc nitrate solution. The mixture was then stirred to homogenize. Under vigorous stirring, to this mixture at 70 °C, 90 mM of NaOH solution was added slowly within 1

h. The reaction mixture was kept under stirring at 70 °C for 2 h. Then, it was left at room temperature overnight to complete precipitation. Thereafter, the composite was cleaned repeatedly with deionized water and separated from the water using a ferromagnet followed by placement in an oven at a temperature of 70 °C for 6 h to dry. After drying, the particles were annealed at a temperature of 350 °C for 1 h.

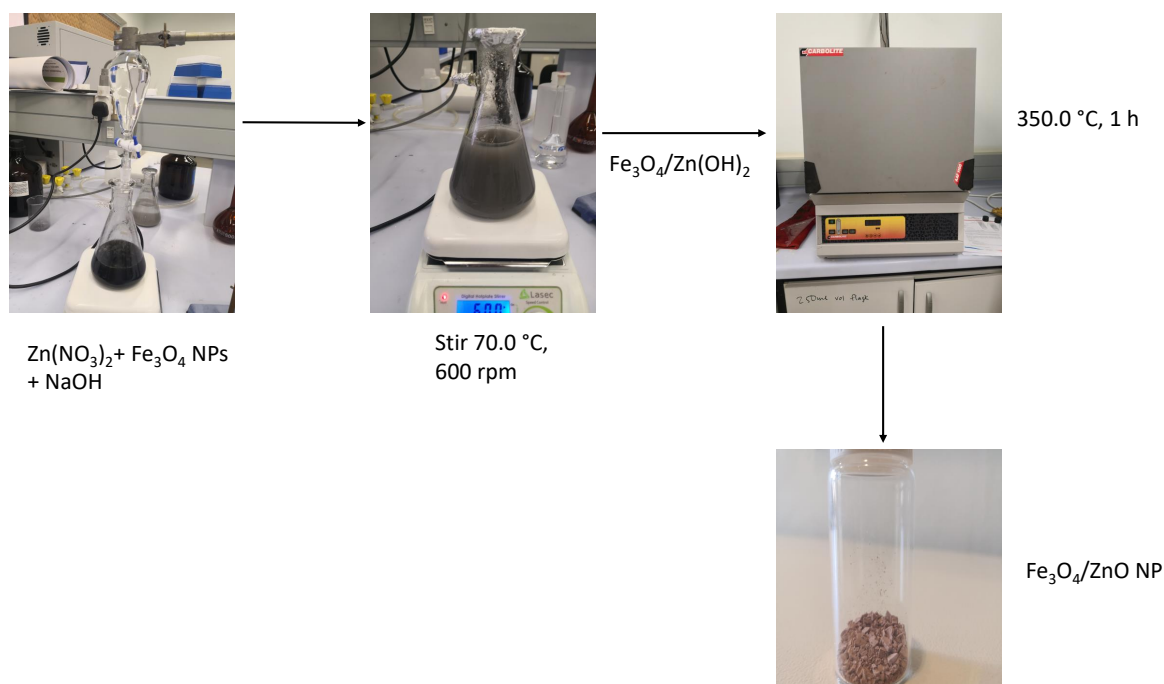


Figure 3.2: Synthesis of ZnO/Fe₃O₄ composite

3.2.1.5 Synthesis of Gold Nanoparticles on ZnO/Fe₃O₄ Composite

A simple but novel thermal method was employed to deposit gold nanoparticles on ZnO/Fe₃O₄ composites as shown on **Figure 3.3**. Briefly, 0.4 g of ZnO/Fe₃O₄ composite was dispersed in 5 mM, 50 mL HAuCl₄ solution (dissolved in ethylene glycol) and sonicated for 3 min to disperse the particles uniformly. The mixture was then placed in a water bath at 95 °C for 20 min. The resulting gold coated ZnO/Fe₃O₄ composites were then cleaned with deionized water and placed in the oven at 70 °C for 3 h.

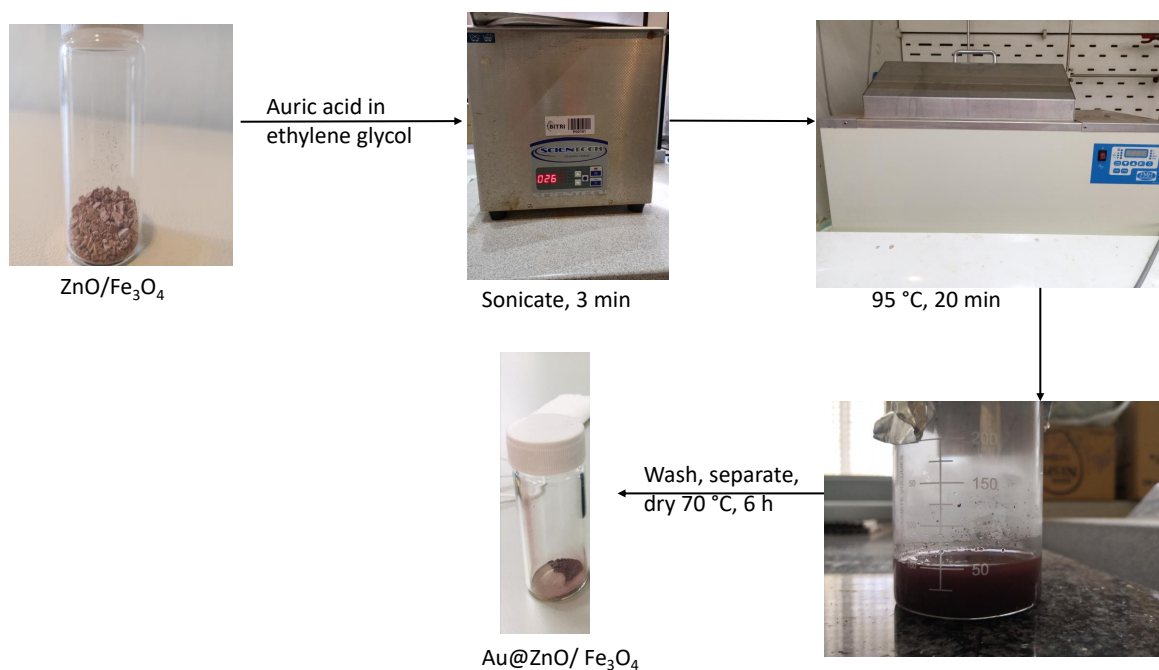


Figure 3.3: Synthesis of Gold Nanoparticles on ZnO/Fe₃O₄ Composite

3.2.1.6 Characterization of the Synthesized Particles

To evaluate and reveal details on the physical and chemical properties of the prepared particles, their SEM micrographs, XRD diffraction patterns, XPS spectra and Raman spectra were obtained.

- **Characterization of the Size, Shape, and Structure of Fe₃O₄, ZnO/Fe₃O₄ and Au@ZnO/Fe₃O₄ Particles**

The size, shape and surface morphology of the Fe₃O₄, ZnO/Fe₃O₄ and Au@ZnO/Fe₃O₄ particles were observed with a high-resolution Carl Zeiss Gemini SEM500 Electron microscope operated at a voltage of 6 kV.

- **Crystalline Structure of the Fe₃O₄, ZnO/Fe₃O₄ and Au@ZnO/Fe₃O₄ Particles**

X-ray diffraction (XRD) patterns of the Fe₃O₄, ZnO/Fe₃O₄ and Au@ZnO/Fe₃O₄ were obtained on a D2 phaser XRD300 W powder diffractometer for a 2θ range of 10–90 at a scan

rate of 0.05 degree/s using Cu K α radiation at 40 kV and 100 mA. The obtained patterns were then compared with standard ones in the database.

- **Confirmation of Formation of Gold Nanoparticles on the Surface of ZnO/Fe₃O₄ by Raman and XPS**

The formation of gold nanoparticles on the surface of ZnO/Fe₃O₄ was confirmed by surface-enhanced Raman spectroscopy (SERS). Raman spectra were measured by Horiba Labram equipped with 532-nm laser line as excitation and a liquid-nitrogen cooled CCD detector. The exposure time was 20 s for the probe molecule. 4-nitrothiophenol (4-NTP) was selected as a probe molecule. The synthesized Fe₃O₄, ZnO/Fe₃O₄ and Au@ ZnO/Fe₃O₄ particles were soaked in 1 mM 4-NTP for 1 h to allow chemisorption of the 4-NTP on the surface of the nanoparticles, then air dried before SERS analysis.

An Axis Supra X-Ray Photoelectron Spectrometer (XPS) was also used to assess the surface chemical composition of the Au@ZnO/Fe₃O₄ and in addition, confirm the formation of an amalgam when Hg²⁺ ions encounter Au. The sample was mounted onto carbon - based conductive adhesive (double sided carbon tape as well as a double sided Cu tape). The samples were then placed into the sample pre-analysis storage in vacuum until it reached 5X10⁻⁷ torr or lower. The samples were then transferred to the analyses chamber (SAC) and left until the vacuum was 5X10⁻⁹ torr or lower before analysis. Wide scans of the samples were carried out to determine the amount of charge compensation, followed by a surface clean with Ar500+ ions for 120 s to remove adsorbed gasses, and finally narrow scans of the elements were carried out.

3.2.1.7 Evaluation of the Reductase-like Activity of the Au@ZnO/Fe₃O₄ Towards 4-Nitrophenol for the Colorimetric Screening of Hg²⁺

To study effect of Hg²⁺ on the catalytic activity of Au@ZnO/Fe₃O₄ composite, the reduction of 4-nitrophenol by NaBH₄ in the presence of Au@ZnO/Fe₃O₄ and in the presence and absence of Hg²⁺ was studied. Here, 3 mg Au@ZnO/Fe₃O₄ was incubated in 100 μ L of 50 μ M

Hg²⁺ solution. Then 1 mL 4-nitrophenol solution (0.1 mM) was added to the solution and 1 mL freshly prepared NaBH₄ solution (100 mM) was quickly added to the mixture. Thereafter, the reactions that were accompanied by a color change were allowed to proceed for 10 min. The resultant solution was scanned by UV/Vis spectrophotometer at a wavelength range of 250-600 nm. For comparison, a control, where 100 μ L deionized water was added to the reaction system instead of Hg²⁺ was also monitored. The absorbance for the reduction of 4-nitrophenol at λ_{max} (λ_{max}) was used as a measure of catalytic activity. A lower absorbance represented a higher catalytic activity and vice versa.

3.2.1.8 Optimization of Incubation Time and Concentration of 4-Nitrophenol for the Detection of Hg²⁺

The effect of incubation time and concentration of 4-nitrophenol on the detection of Hg²⁺ were studied. In a typical experiment, in a quartz cuvette, 3 mg of Au@ZnO/Fe₃O₄ was mixed with 100 μ L of 50 μ M Hg²⁺, then, 1000 μ L of 0.10 mM 4-nitrophenol was added, then, finally, 1000 μ L of 50 mM NaBH₄ was added. The mixture was then incubated for some time. When optimizing the concentration of 4-nitrophenol, increasing concentrations of 4-nitrophenol (0.025-0.125 mM at 0.025 mM intervals) were used. Absorbance readings at each of the concentrations were recorded. When optimizing for time, a reaction mixture that contained the same reagents as when concentration was optimized were used. At the optimized concentration, absorbance readings were taken for 20 min, at 2 min intervals. A control experiment, in which deionized water was used instead of Hg²⁺ was also performed. Absorbance readings for the catalytic reduction of 4-nitrophenol for both the control and samples were recorded at λ ranges of 250-600 nm. All the experiments were performed in triplicates.

3.2.1.9 Extraction of Hg²⁺ From a Skin Lightening Cosmetic Cream

For the analysis of Hg²⁺ in a real sample, 1.0 g of the skin lightening cosmetic cream was weighed accurately into a digestion vessel. A 10 mL mixture of nitric acid and hydrofluoric

acid in the ratio of 4:1 was added to the sample. The mixture vessel was closed and swirled to homogenize the sample with the acid. The mixture was then left for 15 min. Thereafter, the mixture was digested at 200 °C in a microwave digestion system for 15 min to obtain a clear solution. The solution was allowed to cool then transferred to a 100 ml volumetric flask and topped to the mark with distilled water. The skin lightening cosmetic cream extract was spiked at three different concentration levels of the Hg^{2+} and the developed method described in section 3.2.1.7 together with optimized conditions in section 3.2.1.8 were applied to them.

3.2.1.10 Method Validation

Method validation is confirmation via the provision of objective evidence that the requirements for specifically intended use or application of a method have been met. The components of method validation in the present work were linearity, limit of detection (LOD), recovery and precision and selectivity.

- **Linearity**

Linearity of an analytical procedure is its ability to obtain test results that are directly proportional to the concentration of the analyte in the sample within a defined concentration range. Linearity of the method was evaluated by analyzing standard solutions of Hg^{2+} at different concentrations ranging from 0-20 nM. From the data obtained a calibration curve was plotted and the square of the correlation coefficient, R^2 which is a measure of linearity, and the equation of curve were obtained.

- **Limit of Detection (LOD)**

The limit of detection (LOD) of an analytical procedure is the lowest concentration of an analyte in a sample that can be detected by a method but not necessarily quantified as an exact value. In this work, the LOD of the colorimetric sensor was calculated using the equation,

$LOD = 3\sigma/k$, where k is the slope of the calibration curve, and σ is the standard deviation of the blank solution of 4-nitrophenol for the absorbances recorded.

- **Recovery and Precision**

In order to calculate recoveries, the skin lightening cosmetic cream extracts, spiked at three different concentration levels of Hg^{2+} were analysed. Thereafter, percentage recoveries were calculated by comparing absorbance of standard solutions of Hg^{2+} and those of the spiked cosmetic product at the same concentration levels and the corresponding % RSD were also calculated. These recovery experiments were carried out in triplicates ($n=3$). The % RSD and n were taken as a measure of precision.

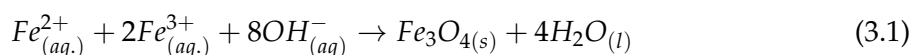
- **Selectivity**

The selectivity of the assay was also a critical parameter for evaluating its performance. The selectivity of the $Au@ZnO/Fe_3O_4$ towards the detection of $50 \mu M Hg^{2+}$ was compared to possible interfering metal ions ($100 \mu M$) including Ag^+ , Na^+ , Cu^{2+} , Mg^{2+} , Ca^{2+} , Cs^{2+} , Zn^{2+} , Pb^{2+} , Co^{2+} , Al^{3+} , K^+ and Ni^{2+} at optimal conditions.

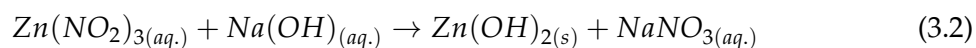
3.2.2 Results and Discussion

3.2.2.1 Fabrication of Fe_3O_4 , ZnO/Fe_3O_4 , and $Au@ZnO/Fe_3O_4$

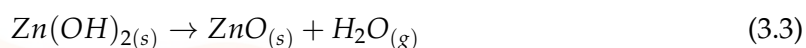
The fabrication of Fe_3O_4 , ZnO/Fe_3O_4 and $Au@ZnO/Fe_3O_4$ was described in sections 3.2.1.3, 3.2.1.4 and 3.2.1.5 respectively. Based on **equation 3.1** below, the formation of a solid state Fe_3O_4 , as the only solid state product meant that the black precipitate that was described in section 3.2.1.3 to have formed on addition of the precipitating agent, aqueous ammonia could be attributed to the Fe_3O_4 solid product in **equation 3.1** below;



The separation of the black precipitate, Fe_3O_4 solid particles by a ferromagnet confirmed the paramagnetism of Fe_3O_4 as has been reported elsewhere [269]. Thereafter, when the precipitate was dispersed in an alkaline zinc nitrate solution, a grey solid powder was formed. Based on **equation 3.2** [270] below, the reaction involved a double exchange that occurred between $\text{Zn}(\text{NO}_3)_2$ and NaOH , resulting in the formation $\text{Zn}(\text{OH})_2$, as the only solid state product.



After annealing the grey solid powder, a brown solid powder was formed and according to **equation 3.3** [270] below, during the annealing process, zinc hydroxide had decomposed into zinc oxide.



The brown solid powder formed during annealing was attracted to a ferromagnet, this in addition confirmed that, a composite of ZnO and Fe_3O_4 which we named, $\text{ZnO}/\text{Fe}_3\text{O}_4$ had been formed and the paramagnetism of the Fe_3O_4 was retained. Finally, a brick-red composite was formed when the brown composite was reacted with auric acid in ethylene glycol at slightly high temperatures, via the polyol synthesis method. The brick-red composite which we named $\text{Au@ZnO}/\text{Fe}_3\text{O}_4$ was also attracted to a ferromagnet, confirming that the paramagnetism of the Fe_3O_4 was still retained. The fabrication of Fe_3O_4 , $\text{ZnO}/\text{Fe}_3\text{O}_4$, and $\text{Au@ZnO}/\text{Fe}_3\text{O}_4$ was confirmed by characterizing for size, shape, morphology, crystallinity and surface chemistry.

3.2.2.2 SEM Characterization

The sizes, shapes and morphologies of the prepared black precipitate attributed to Fe_3O_4 , brown powder attributed to $\text{ZnO}/\text{Fe}_3\text{O}_4$ and brick-red fine particles attributed to $\text{Au@ZnO}/\text{Fe}_3\text{O}_4$ composite were obtained using SEM. The SEM micrograph for Fe_3O_4 displayed generally spherical particles that were mostly agglomerated (see **figure 3.4 a.**), with an average individual particle diameter of 26 nm measured using image J software (see **figure 3.4 d.**). The nano size diameter confirmed the nano particulate nature of the prepared Fe_3O_4 black precipitate.

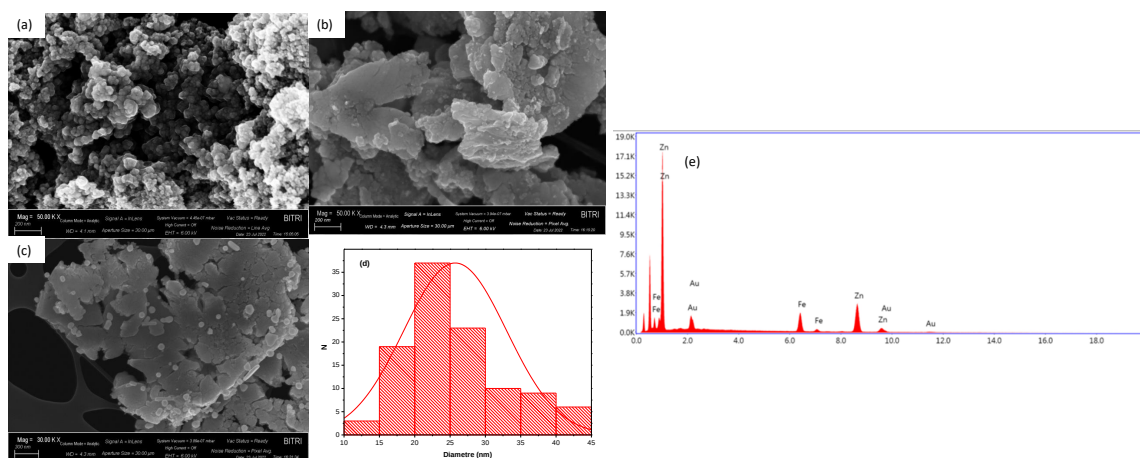


Figure 3.4: SEM images of a. Fe_3O_4 , b. $\text{ZnO}/\text{Fe}_3\text{O}_4$ and c. $\text{Au@ZnO}/\text{Fe}_3\text{O}_4$ nanostructures, d. particle size distribution of Fe_3O_4 e. EDS spectrum of $\text{Au@ZnO}/\text{Fe}_3\text{O}_4$

Our findings were supported by the work of Ba-Abbad et. al. [271], where they reported that using ammonia as a precipitating agent resulted in spherically shaped Fe_3O_4 nanoparticles (NP), whereas using NaOH resulted in cubic shaped NPs. They concluded that the formation of Fe_3O_4 NPs relied on the type of precipitating agent. Independently, Mazhani et. al. [267], Petcharoen and Sinvat [272] also synthesized Fe_3O_4 via co-precipitation method using ammonia as a precipitating agent and similarly, their SEM imaging showed that they had produced spherical shaped Fe_3O_4 NPs. The referenced research works, supported the nano size and spherical shape of Fe_3O_4 particles that we also obtained, with ammonia as the precipitating agent.

Figure 3.4 b. displays the SEM micrograph of the brown $\text{ZnO}/\text{Fe}_3\text{O}_4$ composite. It displays irregular shapes of what looks like broken monoliths. **figure 3.4 c.** is a SEM micrograph of the brick-red fine solid particles of $\text{Au@ZnO}/\text{Fe}_3\text{O}_4$ composite. It displays scarcely dispersed nanoparticles of different sizes and shapes decorated on top of the $\text{ZnO}/\text{Fe}_3\text{O}_4$ monoliths. According to Holade et. al. [273] the reduction of auric acid by ethylene glycol takes place simultaneously with oxidation of ethylene glycol, with several possible oxidation pathways and products (aldehydes, carboxylic acids or CO_2) forming, depending on the temperature, pH and chemical environment. As already established the shapes of the nanoparticles formed are determined by the rate at which metal atoms add to metal clus-

ters (fluxional assemblies) to form seeds (nonfluxional), the structures of these initial seeds (twinned vs single crystal), the rate at which metal atoms add to seed faces, and the binding selectivity of capping agents. The rates at which metal atoms form and assemble are sensitive to reduction kinetics, which in turn are dependent on the reductant [274]. Therefore, we believe that, the combination of moderately high reaction temperature, the solution pH, and ZnO/Fe₃O₄ and Au@ZnO/Fe₃O₄ possibly acting as catalysts in the oxidation of ethylene glycol may have resulted in the formation of different oxidation products or all the possible oxidation products as reductants of Au (III) to Au(0), hence different sizes and shapes of Au (0) forming. The EDS spectrum of Au@ZnO/Fe₃O₄ in **figure 3.4 e.** affirms the presence Au, Zn, and Fe elements in the Au@ZnO/Fe₃O₄, proving the successful formation of Au.

3.2.2.3 UV-Vis Characterization

Figure 3.5 shows the absorption spectrum of Au@ZnO/Fe₃O₄. The spectrum clearly illustrates two peaks; one at the UV region around 361 nm and the other in the visible region around 519 nm. The peak in the UV region is due to the band edge absorption of ZnO and that in the visible range is due to the surface plasmon absorption of Au NPs, confirming the existence of the Au particles in nano-size [275].

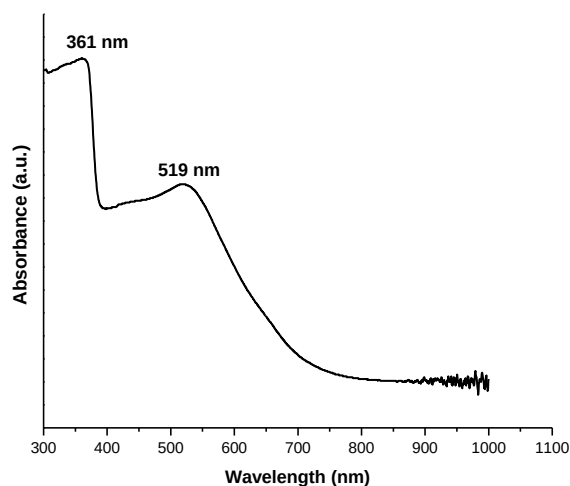


Figure 3.5: UV-Vis absorption spectrum of Au@ZnO/Fe₃O₄

3.2.2.4 XRD Characterization

Analysis through X-ray diffraction spectrophotometry was carried out to confirm the crystalline nature of the fabricated particles. The X-ray diffraction (XRD) pattern marked Fe_3O_4 shown in **figure 3.6** exhibited 2θ peaks at 30.20° , 35.45° , 43.25° , 53.55° , 56.78° and 62.73° marked with indices at (220), (211), (400), (422), (511) and (400) (JCPDS No. 19-0629) respectively. The peak positions and intensities matched well with those reported before for inverse spinel structure of magnetite [276, 277] and have been reported to show the face centered cubic structure of Fe_3O_4 [264].

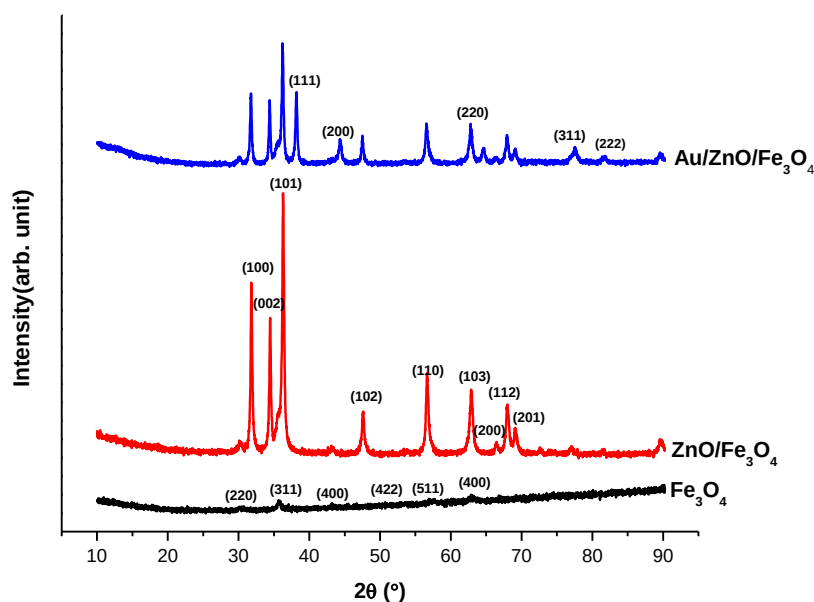


Figure 3.6: XRD patterns of Fe_3O_4 , $\text{ZnO}/\text{Fe}_3\text{O}_4$, and $\text{Au@ZnO}/\text{Fe}_3\text{O}_4$

Furthermore, on the XRD pattern marked $\text{ZnO}/\text{Fe}_3\text{O}_4$ in **figure 3.6**, the peaks at 2θ 31.77° , 34.42° , 36.25° , 47.54° , 56.60° , 62.86° , 66.38° , 67.96° and 69.10° corresponding to (100), (002), (101), (102), (110), (103), (200), (112) and (201) (JCPDS No. 36-1451) respectively were observed. The positions and relative intensities of the diffraction peaks matched well with those reported before for hexagonal zincite-type of structure [278, 279] which indicated the formation of a highly crystalline ZnO structure [280]. In this pattern, in addition to the peaks assigned to ZnO, the peaks of cubic spinel structured magnetite were observed in the nanocomposites, revealing that the phases of Fe_3O_4 nanocrystals did not change, further-

more, this confirmed the formation of ZnO/Fe₃O₄ composites.

Finally, on the XRD pattern marked Au@ ZnO/Fe₃O₄, five strong peaks that were not observed for ZnO/Fe₃O₄ composite appeared. The peaks were observed at diffraction angles of 38.20°, 44.40°, 64.60°, 77.60° and 81.76° with the indices (111), (200), (220), (311) and (222) (JCPDS No. 04-0784) respectively. The peaks have been attributed to the Au crystal with a cubic phase [281, 282]. The peaks attributed to ZnO structure still appeared on this pattern. The XRD results clearly indicated the successful synthesis of Au@ZnO/Fe₃O₄ hybrid material.

3.2.2.5 Raman Characterization

The formation of gold nanoparticles on the surface of ZnO/Fe₃O₄ was confirmed by surface-enhanced Raman spectroscopy (SERS). Gold nanoparticles can produce ultrasensitive surface-enhanced Raman scattering (SERS) signals owing to their plasmonic properties. Collective oscillation of free electrons on the surface of gold nanoparticles enhance the Raman signal of molecules which are adsorbed or in close proximity to gold nanoparticles [283]. The mechanism relevant to SERS is the surface enhancement of the electromagnetic field at the interface between the SERS active substrate (e.g. gold) and a molecule of interest through resonant excitation of surface plasmons of the metal [284]. This mechanism was exploited to confirm the formation of AuNPs on the surface of ZnO/Fe₃O₄. SERS effect of the nanostructures were investigated using 4-Nitrothiophenol (4-NTP) as a probe molecule because of its strong Raman activity and strong affinity to chemisorption on gold surfaces [285]. The synthesized Fe₃O₄, ZnO/Fe₃O₄ and Au@ ZnO/Fe₃O₄ particles were soaked in 1 mM 4-NTP for 1 h to allow chemisorption of the 4-NTP on the surface of the nanoparticles then air dried before SERS analysis. The SERS spectrum on **figure 3.7** labeled Au@ ZnO/Fe₃O₄ showed bands at 1567 cm⁻¹, 1336 cm⁻¹, 1110 cm⁻¹, 1075 cm⁻¹, 854 cm⁻¹ and 722 cm⁻¹ for 4-nitrothiophenol. These could be attributed to the benzene ring C=C stretching, symmetrical NO₂ stretching, C-H bending vibration, in-plane C-S stretching modes, C-H wagging vibrations and C-S wagging vibrations, respectively [267, 286, 287]. On the other hand, Raman scattering was

not observed under the measured conditions for ZnO/Fe₃O₄ and Fe₃O₄ as shown on **figure 3.7**, indicating that the particles did not exhibit any SERS effect. This served as confirmation that gold nanoparticles were formed on the surface of ZnO/Fe₃O₄.

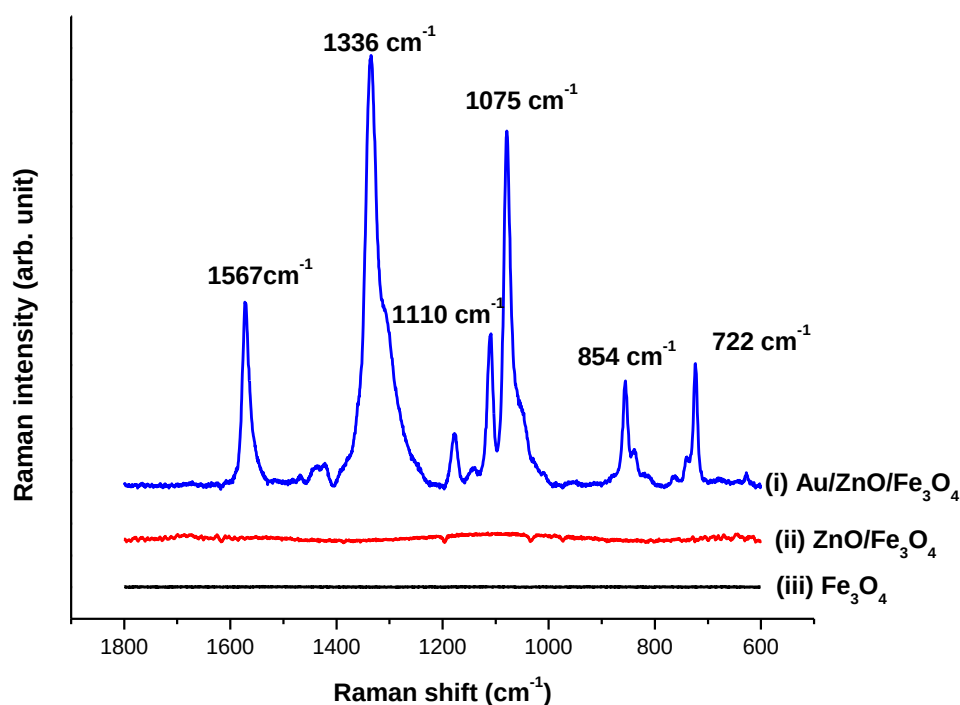


Figure 3.7: SERS spectra of pNTP with (i) Au@ZnO/Fe₃O₄ (ii) ZnO/Fe₃O₄ and (iii) Fe₃O₄ as SERS substrates

3.2.2.6 XPS Characterization

XPS analysis was carried out to assess the surface chemical composition of the Au@ZnO/Fe₃O₄.

Figure 3.8 a. illustrate Zn 2p XPS spectra of Au@ZnO/Fe₃O₄ illustrate perfect fit for two distinct peaks of Zn 2p_{3/2} and Zn 2p_{1/2} located at the binding energy of 1023.40 and 1046.43 eV, respectively, pertain to Zn²⁺ cations [288]. Moreover, a spin-orbital splitting in Zn 2p energy level of 23.03 eV was observed. This means that Zn 2p split into of binding energy separation of 23.03 eV between the Zn 2p_{3/2} and Zn 2p_{1/2} core levels and reflects the presence of oxidized Zn (ZnO) and not metallic Zn (Zn⁰) [289]. **Figure 3.8 b.** illustrates the deconvolution of O 1s XPS spectra, with three separate peaks at the binding energy of 532.00, 533.30

and 534.40 eV. The band at 532.00 eV is attributed to lattice oxygen in wurtzite ZnO forming Zn-O bonding, while the band at 533.30 eV is attributed to O^{2-} ions in oxygen-deficient regions within the ZnO matrix (oxygen vacancies) and the surface adsorbed loosely bonded oxygen like hydroxyls (OH) bonds, i.e., ZnO(OH). The band at 534.40 eV can be ascribed to the specific chemisorbed oxygen, from adsorbed CO_2 , O_2 , or H_2O [290, 291]. The XPS spectrum in **figure 3.8 c.** shows overlapping peaks. Upon deconvolution, it resolved into four peaks at binding energies 93.18 eV, 90.51 eV, 88.82 eV and 85.07 eV. These peaks were attributed to Zn $3p_{1/2}$, Zn $3p_{3/2}$, Au $4f_{5/2}$ and Au $4f_{7/2}$, respectively [232, 292]. A spin-orbit splitting value, 3.75 eV, i.e., the separation binding energy values between Au $4f_{7/2}$ and Au $4f_{5/2}$ core-level excited photoelectron lines was observed, this matched very well with the spin-orbit-splitting values from bulk reference gold samples reported in literature [293], this confirmed the metallic state of Au in the hybrid material. The spectrum obtained confirmed the formation of Au NP on the surface of ZnO.

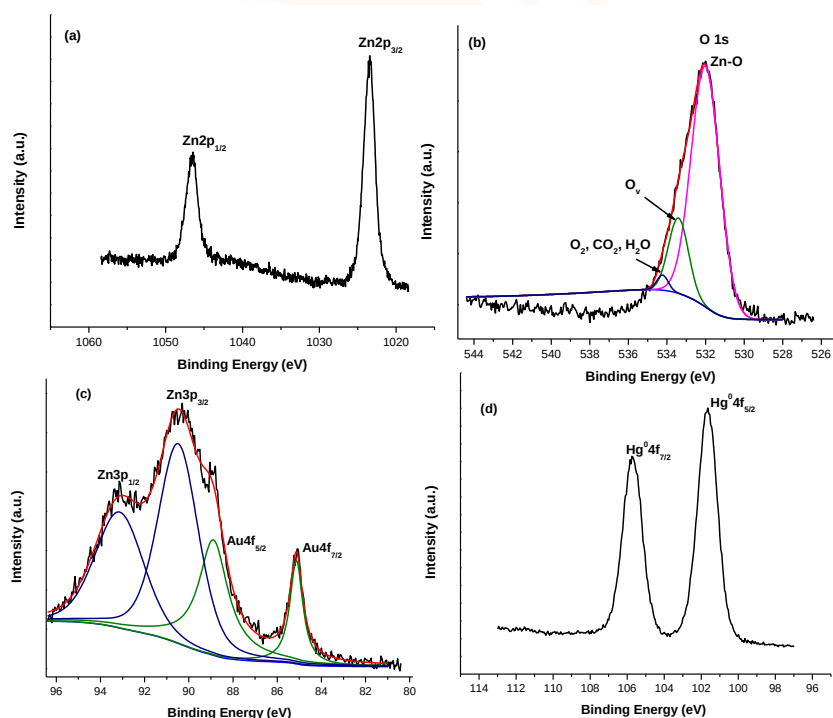


Figure 3.8: XPS spectra of a. Zn 2p, b. O 1s, c. Au 4f and Zn 3p regions of Au@ZnO/Fe₃O₄ hybrid, d. XPS spectrum of Hg 4f region of Au@ZnO/Fe₃O₄ hybrid after interaction with Hg²⁺

The XPS spectrum in **figure 3.8 d.** shows two binding-energy peaks at 101.64 eV and 105.64 eV that were formed on addition of Hg^{2+} . The peaks were ascribed to $\text{Hg } 4f_{5/2}$ and $\text{Hg } 4f_{7/2}$, respectively [50] which revealed that Hg^0 was formed on the surface of $\text{Au@ZnO/Fe}_3\text{O}_4$, confirming the formation on an Au-Hg amalgam on the surface of $\text{Au@ZnO/Fe}_3\text{O}_4$ as has been reported by Long et. al. [239] in the past. In the same study, Long et. al. further reported that the existence of Hg^0 on the surface of AuNPs had great influence on the features of the surface of AuNPs, including adsorption on the surface of AuNPs.

3.2.2.7 Catalytic Performance of the Prepared $\text{Au@ZnO/Fe}_3\text{O}_4$ Hybrid Material

The excellent qualities of the different components that constituted the prepared catalytic hybrid material, $\text{Au@ZnO/Fe}_3\text{O}_4$ allowed versatility in the design and tailoring of a multi-functional material with distinct properties that were brought about by the combined effect of the traits of the individual components. For instance, the prepared Fe_3O_4 nanoparticles provided the magnetic separation property, however the ferromagnetic NPs aggregated too easily due to their magnetic dipole-dipole interactions and decreased the efficiency of the catalytic property of NPs [50]. Also, according to Chen et. al. [294] it is notably difficult to directly attach Au NPs to the surfaces of the Fe_3O_4 owing to the dissimilar nature of the two species' surfaces. To deal with this challenge, we successfully used ZnO NPs to facilitate the formation of AuNP on the nanomaterial because of the closeness of intrinsic absorption and emission bands of ZnO with the surface plasmon peak of Au at resonant coupling.

In this work, the reduction of 4-nitrophenol to 4-aminophenol in the absence and presence of the prepared $\text{Au@ZnO/Fe}_3\text{O}_4$ as a catalyst as well as in the absence and presence of Hg^{2+} as the analyte of interest, was monitored via UV-visible spectroscopy which showed differences in absorbance intensities at $\lambda_{\text{max}} = 400 \text{ nm}$ in the different reaction systems (see **figure 3.9**). **Figure 3.9 i.** shows that the recorded absorbance for the reduction of 4-nitrophenol by excess NaBH_4 solution in the absence of the catalyst was highest and was found to be lowest in the presence of the catalyst (see **figure 3.9 ii.**). In the presence of the catalyst, AuNPs acted as electron relay centers and initiated the shifting of electrons from

the donor, BH_4^- to the acceptor, 4-nitrophenol. The reacting molecules (BH_4^- ion and 4-nitrophenol) were simultaneously adsorbed onto the surface of the NPs. As a result, electrons were transferred from the BH_4^- ion into 4-nitrophenol through the NPs, causing the fast reduction of 4-nitrophenol to 4-aminophenol [295] and the lowest absorbance recorded. Without the catalyst, the reduction of 4-nitrophenol by excess NaBH_4 was very slow hence very little 4-nitrophenol was converted to 4-aminophenol and the absorbance was highest.

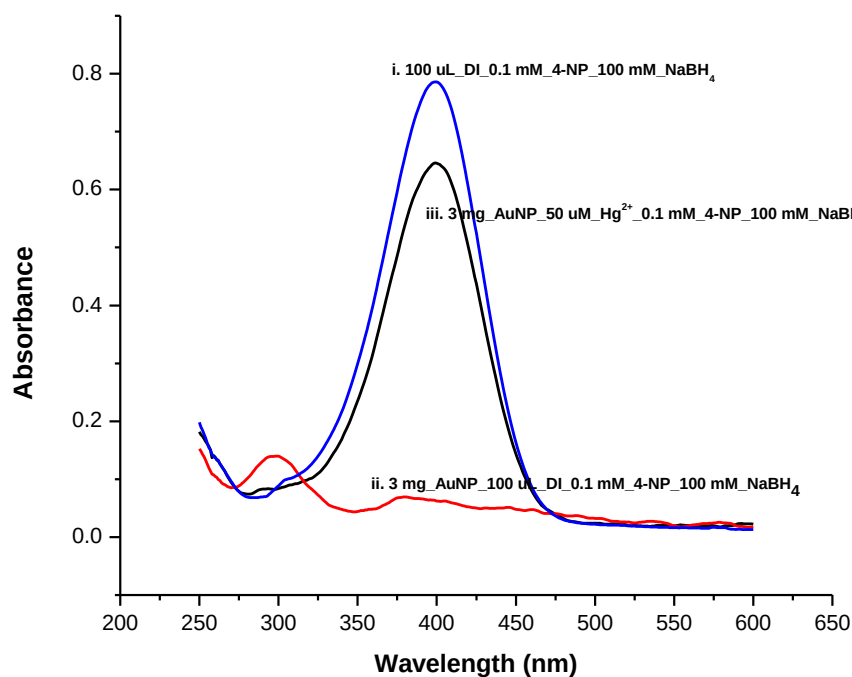


Figure 3.9: UV-vis absorbance spectra showing the effect of the catalyst and Hg^{2+} on the reduction of 4-nitrophenol to 4-aminophenol

However, when Hg^{2+} was added to the system (see **figure 3.9 iii.**), the absorbance at 400 nm was reduced but only to a lesser extent compared to when Hg^{2+} was not added to the system (**figure 3.9 ii.**), depicting a decrease on the catalytic activity of the $\text{Au@ZnO/Fe}_3\text{O}_4$. This reduction in catalytic activity was because of the inhibition caused by the formation of gold-hydride species by the resulting Au-Hg amalgam formed, leading to the slow reduction of 4-nitrophenol to 4-aminophenol. The formation of a Au-Hg amalgam which causes the slow reduction of 4-nitrophenol to 4-aminophenol has also been reported before by He et. al. [296]. A schematic illustration of the reaction for the effect of Hg^{2+} on the catalytic reduction of 4-nitrophenol reaction which represents the main principle for this work is shown on

figure 3.10. It should be noted that the observations in **figure 3.9** were accompanied by color changes. The reaction system attributed to the reduction of 4-nitrophenol by excess NaBH_4 in the absence of the catalyst represented by the spectrum in **figure 3.9 i.** was bright yellow in color and then when the catalyst was added to the system represented by the spectrum in **figure 3.9 ii.** the solution changed to colourless and finally when mercury was added to the system represented by the spectrum in **figure 3.9 iii.** the solution only changed to pale yellow. The color changes, with further investigations may be employed in the colorimetric screening of Hg^{2+} .

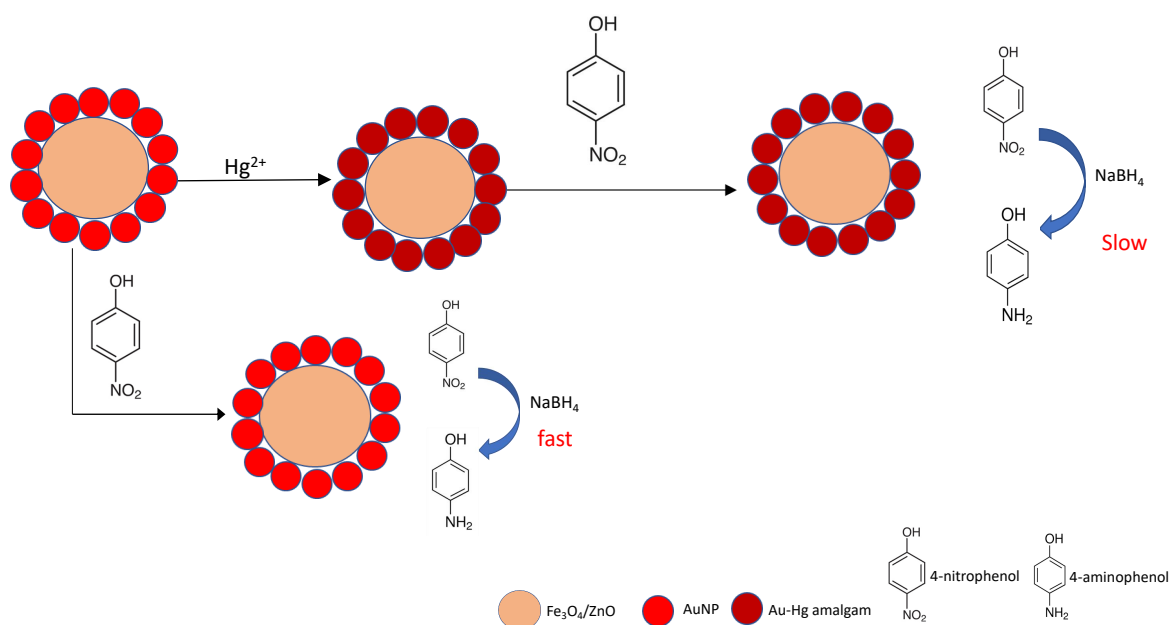


Figure 3.10: Schematic illustration of $\text{Au@ZnO/Fe}_3\text{O}_4$ colorimetric probe for screening of Hg^{2+} ions

3.2.2.8 Optimization of Incubation Time and Concentration of 4-NP for the Detection of Hg^{2+}

The incubation time for the colorimetric screening of Hg^{2+} via the catalytic reduction of 4-nitrophenol to 4-aminophenol was studied in the absence and presence of mercury in order to identify the best time at which the reaction occurs. The results are shown on **figure 3.11**.

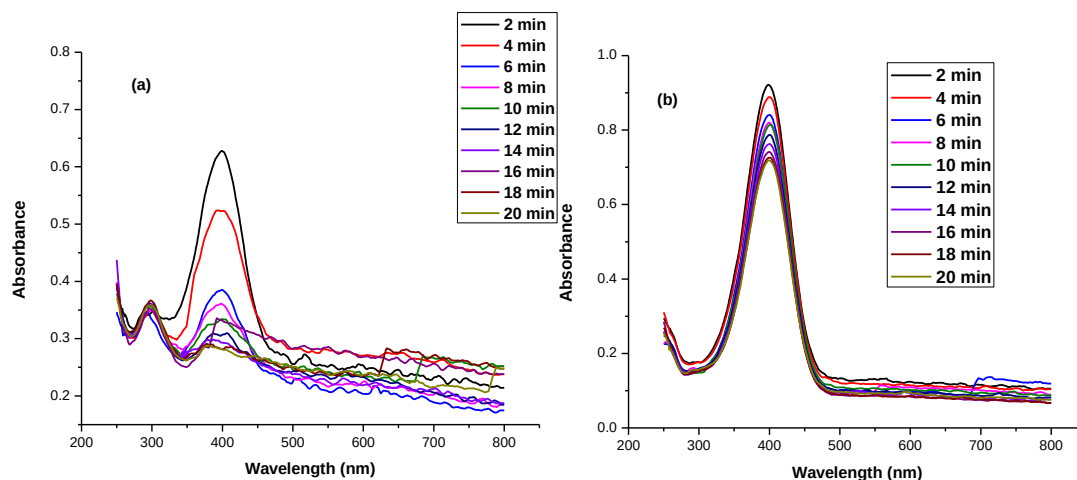


Figure 3.11: The effect of incubation time on the response signal; Spectra showing the effect of incubation time on the response signal of 0.1 mM 4-nitrophenol in the sensing system at 400 nm a. before and b. after adding mercury.

The spectra on **figure 3.11 a.** and **b.** show a decrease in the absorption signal at $\lambda_{\text{max}} = 400 \text{ nm}$ with increasing time both in the absence and presence of Hg^{2+} respectively, signaling the reduction of 4-nitrophenol to 4-aminophenol with time. This reaction was slower in the presence of Hg^{2+} than in its absence, due to an increase in the quantity of Au-Hg amalgam formed with increase in time. The experiment was stopped at 20 mins since there was no discernible absorbance peak at $\lambda_{\text{max}} = 400 \text{ nm}$ in the absence of Hg^{2+} . From the spectra in **figure 3.11 a.** it was concluded that a reasonable time at which this experiment could be carried out showing discernible peaks was any time between 8 and 12 min. Thus, for our work 10 mins was chosen as the optimal time. It should be noted that there were corresponding color changes with time that were also observed.

The effect of concentration of 4-nitrophenol on the absorbance signal was also studied. The results are shown on **figure 3.12.**

As depicted by the spectra on **figure 3.12 a.** and **b.**, the absorbance increased with increasing concentration of 4-nitrophenol at optimal 10 min incubation time, both in the absence and presence of Hg^{2+} respectively. In both cases there were more 4-nitrophenol molecules in the system available to be reduced, hence the increase in absorbance with increasing concentrations of 4-nitrophenol. There were discernible peaks at all concentration levels, therefore

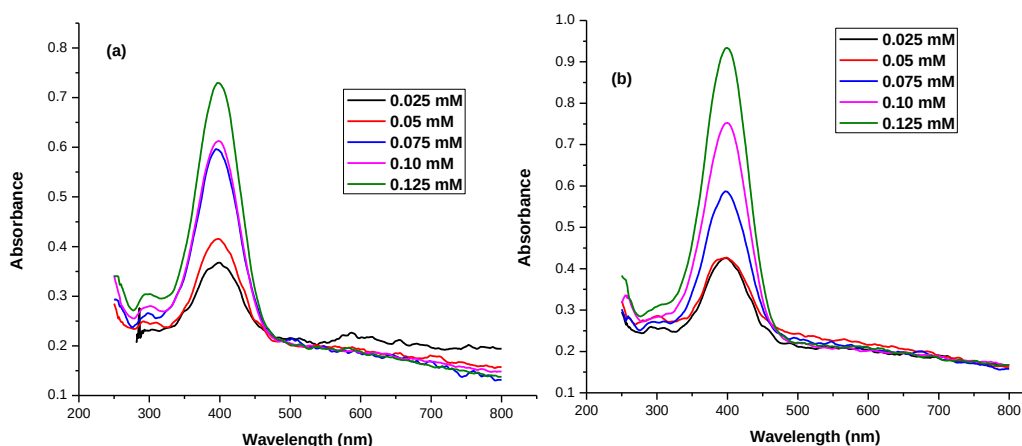


Figure 3.12: The effect of the amount of 4-nitrophenol on the response signal; Spectra showing the effect of varying concentrations of 4-nitrophenol on the response signal a. before and b. after adding mercury.

any of the concentrations of 4-nitrophenol studied was suitable for the colorimetric screening of Hg^{2+} to give distinct color changes. Thus for this work 0.05 mM 4-nitrophenol was used.

3.2.2.9 Method Validation

- **Linearity**

The linearity for the colorimetric probe was studied at optimal conditions. **Figure 3.13 a.** shows the absorbance spectra for the catalytic reduction of 0.05 mM 4-nitrophenol in the presence of increasing concentrations of Hg^{2+} (0-20 nM) after incubating for 10 min. It shows increasing absorbance at 400 nm with increasing concentration of Hg^{2+} .

Figure 3.13 b. presents the absorbance signal difference as a function of Hg^{2+} concentration. In the graph, the absorbance difference increased with increasing concentration of Hg^{2+} until 16 nM then began to decrease. The absorbance signal was linearly related to Hg^{2+} concentration in the range of 0-10 nM with an R^2 value of 0.9914. The value of R^2 obtained meant that for the concentration range of 0-10 nM there was a strong linear correlation between the concentration of the Hg^{2+} and the intensity. From this it can be concluded that the method was valid in the concentration range 0-10 nM for the detection of Hg^{2+} . Compared

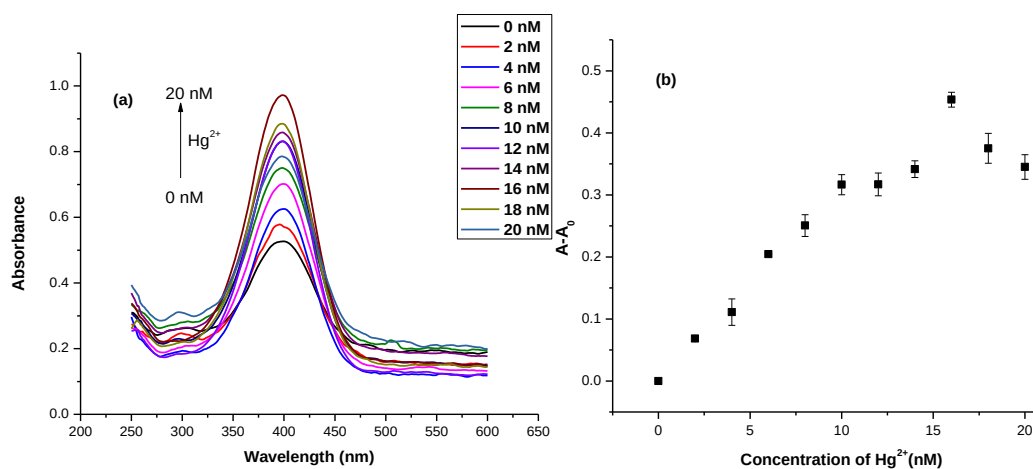


Figure 3.13: Sensitivity of the colorimetric probe, Au@ZnO/Fe₃O₄ towards the screening of Hg²⁺: a. absorption spectra for the catalytic reduction of 4-nitrophenol under varying concentrations of Hg²⁺ (0-20 nM), b. calibration curve of absorbance variation versus the concentration of Hg²⁺ added

to other colorimetric methods employing Au-based nanozymes (**table 3.1**) the current proposed method fairs well. Not only does it offer high sensitivity and fast response, but it is also convenient and stable.

Material	Method	Linear range (nM)	Detection limit (nM)	Ref
AuNP@MOF	Reductase mimetic	0.2-0.4	0.10	[232]
AuNP	Reductase mimetic	0-10	1.45	[239]
Au@ZnO/Fe ₃ O ₄	Reductase mimetic	0-10	2.34	This worked
Fe ₃ O ₄ @ZnO	Peroxidase mimetic	0-10	23	[277]
Cs-AuNP	Peroxidase mimetic	0-200	20	[51]

Table 3.1: comparison of the proposed method with other reported Au-based nanozymes for the detection of Hg²⁺

• The limit of Detection

The LOD of the colorimetric probe was calculated using the equation, $LOD = 3\sigma/k$, where k is the slope of the calibration curve, and σ is the standard deviation of the blank solution of 4-nitrophenol absorbance recorded. It was calculated to be as low as 2.34 nM (4.694×10^{-4} ppm). This LOD was very low and allowed for trace determination of mercury in cosmetic products below the stipulated USFDA regulatory limit of 1 ppm.

- **Recovery, Precision and Determination of Mercury in Real Samples**

The performance of the proposed colorimetric method was evaluated for the determination of mercury in a cosmetic product purchased locally. The results are summarized in **table 3.2**. As shown on the table, excellent recoveries in the range of 96.5 -100.3 % with low %RSDs in the range 4.3-13.8 % for n=3 were obtained. The high recoveries and low %RSDs indicated that the proposed method had high accuracy and precision for the analysis of Hg^{2+} in real samples, respectively.

Spiking concentration (nM)	Detected concentration (nM)	Recovery %	RSD %
4.00	4.01	100.29	13.83
6.00	5.93	98.78	7.96
8.00	7.72	96.49	4.26

Table 3.2: The detection of Hg^{2+} in a cosmetic product

- **Selectivity of the $\text{Au@ZnO/Fe}_3\text{O}_4$ Towards the Detection of Hg^{2+} for the Proposed Colorimetric Method**

The unique affinity of Hg^{2+} towards Au provided excellent selectivity towards detecting over other potentially interfering metal ions. **Figure 3.14** show that other metal ions including Ag^+ , Na^+ , Cu^{2+} , Mg^{2+} , Ca^{2+} , Cs^{2+} , Zn^{2+} , Pb^{2+} , Co^{2+} , Al^{3+} , K^+ and Ni^{2+} did not have the ability to suppress the catalytic reduction of 4-nitrophenol by $\text{Au@ZnO/Fe}_3\text{O}_4$, instead they seemed to enhance it at double the concentration of Hg^{2+} .

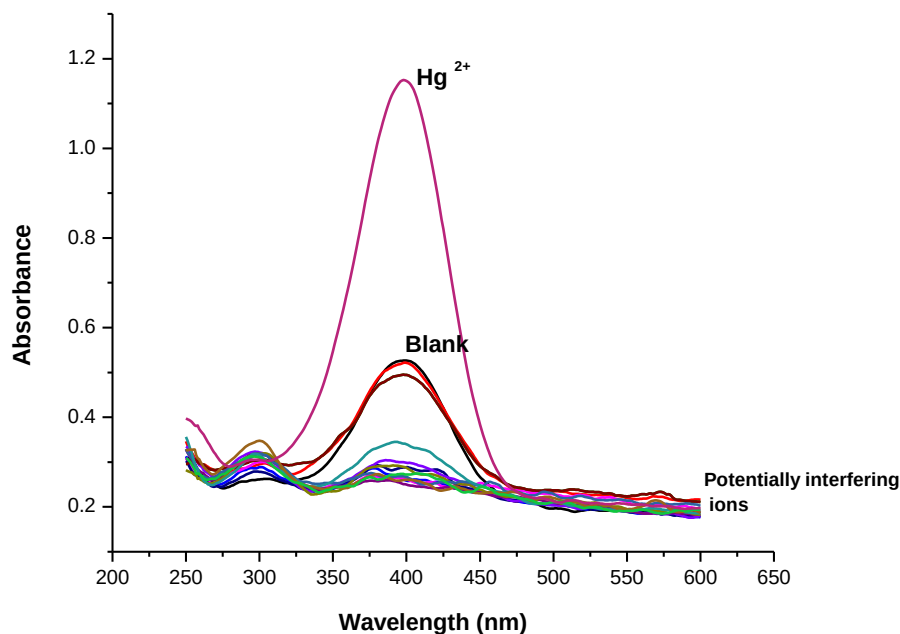


Figure 3.14: Selectivity of the colorimetric probe, Au@ZnO/Fe₃O₄ towards the screening of Hg²⁺: absorption spectra for the catalytic reduction 4-nitrophenol in the presence of Hg²⁺ and possible competing species.

3.3 Morula Leaf Extract Mediated Synthesis of Silver Nanoparticles and Their Application in the Analysis of SCN⁻ Based on Inhibition of the Nanozyme Activity on 3,3',5,5'- Tetramethylbenzidine (TMB) by the Silver Nanoparticles

3.3.1 Materials and Methods

3.3.1.1 Chemicals

The silver nitrate used for preparing the nanoparticles was purchased from Merck (Darmstadt, Germany). Cesium nitrate, lead (III) nitrate, ascorbic acid, L-lysine, cysteine, and glutathione were purchased from Sigma Aldrich (Johannesburg, South Africa). Aluminum nitrate nonahydrate and ferric chloride hexahydrate were purchased from Ace Chemicals (Johannesburg, South Africa). Fructose and glucose were purchased from Saarchem (Johannesburg, South Africa). Calcium chloride dihydrate, magnesium nitrate hexahydrate, potassium sulphate, sodium sulphite, and ferrous sulphate were purchased from Rochelle (Johannesburg, South Africa). Sodium nitrate, zinc nitrate, potassium thiocyanate, methanol,

sodium phosphate dibasic hydrate, and citric acid were purchased from Merck (Darmstadt, Germany). A 500 mL milk sample was purchased from a local supermarket (Palapye, Botswana). Marula leaves were harvested from a tree in BIUST campus (Palapye, Botswana). All reagents were of analytical grade and were used without additional purification. All analyte solutions were prepared in Millipore ultrapure double deionized water.

3.3.1.2 Instrumentation

The morphology of the prepared AgNPs was observed using the Carl Zeiss Gemini SEM500 Electron microscope purchased from Carl Zeiss (Berlin, Germany). X-ray diffraction (XRD) patterns were obtained on an Empyrean x-ray diffractometer supplied by PANalytical (Malvern, United Kingdom) for a 2θ range of 20–80 at scan rate of 0.05 degree/s using Cu K α radiation at 40 kV and 100 mA. A UV-visible absorbance spectrophotometer purchased from Thermo-fisher (Waltham, MA, USA) was used to collect UV-vis spectra using 1 cm glass cuvettes as sample cells. A pestle and mortar supplied by the Chemical and Forensic Science Department in BIUST (Palapye, Botswana) was used to grind dried marula leaves.

3.3.1.3 Synthesis of Sclerocarya Birrea Morula Extract Reduced Silver Nanoparticles

Silver nanoparticles were prepared from Sclerocarya birrea leaf extracts using a method by Nyoni et al. [297] with some modifications as shown by schematic representation of the reaction on **Figure 3.15**. Sclerocarya birrea leaves were washed three times with deionized water to remove dust and impurities. The leaves were air dried in the shade at room temperature. The dried leaves were ground into fine powder. 5 g of the ground leaf powder was boiled at 120 °C in 50 mL deionized water for about 5 min while continuously stirring. The extract was cooled to room temperature and filtered thrice through a Whatman number 1 filter paper. For the preparation of the silver nanoparticles, 150 μ L of the extract was added to 100 mL, 5 mM AgNO₃ while stirring. The reaction progress was monitored by taking 2 mL of the reaction mixture for absorbance measurements every 15 min for a period 3 h using a UV-vis spectrophotometer in the wavelength ranges of 300 to 800 nm.

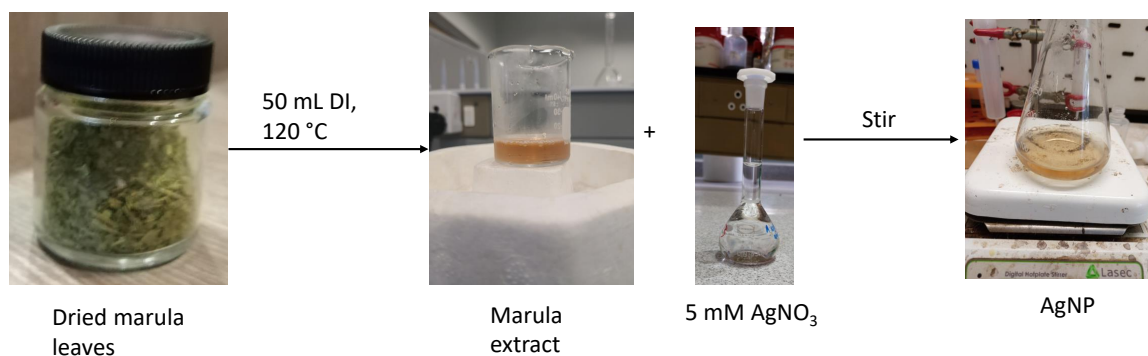


Figure 3.15: Synthesis of *Sclerocarya birrea* morula extract reduced silver nanoparticles using silver nitrate

3.3.1.4 Characterization of the *Sclerocarya Birrea* Extract Bio-synthesized AgNPs

To evaluate and reveal details on the physical and chemical properties of the *Sclerocarya birrea* extract prepared particles, their SEM micrographs and XRD diffraction patterns were obtained. The size, shape and surface morphology of the bio-synthesized particles were observed with a high-resolution Carl Zeiss Gemini SEM500 Electron microscope operated at a voltage of 3 kV. To study the crystallinity of the bio-synthesized AgNPs, their X-ray diffraction (XRD) pattern was obtained on a Empyrean x-ray diffractometer for a 2 range of 20–80 at a scan rate of 0.05 degree/s using Cu K radiation at 40 kV and 100 mA. The obtained pattern was then compared with a standard bio-synthesized AgNPs pattern in the database.

3.3.1.5 Evaluation of the Oxidase-like Activity of the AgNPs Towards TMB for the Colorimetric Screening of SCN[−]

The oxidase-like activity of the AgNPs and their significance in the colorimetric detection of SCN[−] was studied with TMB as a chromogenic substrate. Several reaction mixtures that included; TMB alone, TMB and AgNPs, TMB and SCN[−], TMB, SCN[−] and AgNPs, were prepared in citrate-phosphate buffer and UV/visible absorption spectroscopy measurements were carried out at λ ranges of 250 to 800 nm to assess oxidase-like activity in these reaction mixtures. The absorbance intensities at λ_{max} and the corresponding color changes for these reaction systems were used to assess the oxidase-like activity in the reaction mixtures. A

higher absorbance represented a higher catalytic activity and vice versa.

3.3.1.6 Optimization of Parameters for the Detection of Thiocyanate

To achieve high efficiency in the detection of thiocyanate, a series of parameters were studied, these included, volume of silver nanoparticles, concentration of TMB, incubation time and buffer pH. In a typical experiment, in a 2 mL eppendorf tube, increasing volumes Ag-NPs were mixed with 50 μL of thiocyanate then 1000 μL of citrate-phosphate buffer and 100 μL of TMB were added. The mixture was then incubated for 3 min. A control experiment in which deionized water in the absence of thiocyanate was also performed. After incubation, absorbances were recorded at λ_{max} in the wavelength ranges of 250 to 800 nm. All the experiments were carried out in triplicates ($n=3$). In each case the parameter under study was varied. The results obtained were used to construct plots of absorbance versus volume of silver nanoparticles, concentration of TMB, incubation time or buffer pH both in the presence and absence of the analyte, SCN^- . Finally, the optimal parameters were taken as a point on the graph where the absorbance differences between absorbance in the presence and absence of the analyte were maximum.

3.3.1.7 Extraction of SCN^- From a Milk Sample

A simple method based on methanol deproteinization was used for the preparation of milk samples for SCN^- analysis. 0.50 g of milk was filled up to 10 mL with methanol. The solution was then centrifuged at 3,000 rpm for 10 min at 5 $^{\circ}\text{C}$. From this, the supernatant was diluted five times with water then filtered with 0.4 μm filter. 30 μL SCN^- of different concentrations was added to 270 μL of the milk extract solution to make up final concentrations of SCN^- at 10, 15 and 30 μM . From these, 50 μL was used for analysis.

3.3.1.8 Method Validation: Efficacy of the Method in the Determination of SCN^- in Milk

The components of method validation in the present work were linearity, limit of detection (LOD), recovery and precision and selectivity.

- **Linearity**

To evaluate the linearity of the method in the detection of SCN^- , under optimal conditions, SCN^- solutions of varying concentrations (0-1000 μM) were added to the fabricated colorimetric probe and the corresponding absorbances were recorded at λ_{max} . From the spectra obtained, a calibration curve of absorbance versus concentration of SCN^- was plotted and the square of the correlation coefficient, R^2 was obtained.

- **Limit of Detection (LOD)**

In this work, the LOD of the colorimetric sensor is calculated using the equation, $\text{LOD} = 3\sigma/k$, where k is the slope of the calibration curve, and σ is the standard deviation of the blank solution of TMB for the absorbances recorded.

- **Precision and Recovery**

To calculate recoveries, the milk extracts, spiked at three different concentration levels of SCN^- were analyzed. Thereafter, percentage recoveries were calculated by comparing absorbances of the standard solutions of SCN^- and those of the spiked milk extract at the same concentration levels and the corresponding %RSD were also calculated. These recovery experiments were carried out in triplicates ($n=3$). The percentage of the relative standard deviation (%RSD) was taken a measure of precision.

- **Evaluation of the Selectivity of the AgNPs Nanozyme**

To assess selectivity, some competing species were used instead of SCN^- . To expand the field of application, SCN^- was analyzed in the presence of the competing species at equal concentrations (100 μM).

3.3.1.9 Steady State Kinetics

Under optimum conditions, the steady-state kinetics of the enzyme mimetic towards the oxidization of TMB with dissolved oxygen were determined. Here the absorbance intensities of increasing concentrations of TMB (2.0 – 10.0 mM) in the presence of the AgNPs were recorded at 1 min intervals for 5 min.

3.3.2 Results and Discussion

3.3.2.1 Bio-synthesis of *Sclerocarya birrea* (Morula) Extract Reduced Silver Nanoparticles

AgNPs were prepared by the method described in section 3.3.1.3. After the plant extract was added, the clear silver nitrate solution changed to a yellowish brown solution. From the UV-Visible spectra obtained, **figure 3.16**, there was a peak at $\lambda_{\text{max}} = 430 \text{ nm}$, this was attributed to the surface plasmonic resonance peak for AgNPs as observed by previous reports by Kaur et. al. [298]. Jian et.al. [299] concluded that this peak indicated the successful synthesis of AgNPs and was due to strong absorption of visible light due to excitation of the nanoparticles' surface plasmons. The absorbance for silver nanoparticles increased with increasing reaction time indicating an increase in AgNPs yield with time. This is in agreement with previously reported work by Nyoni et. al. [297].

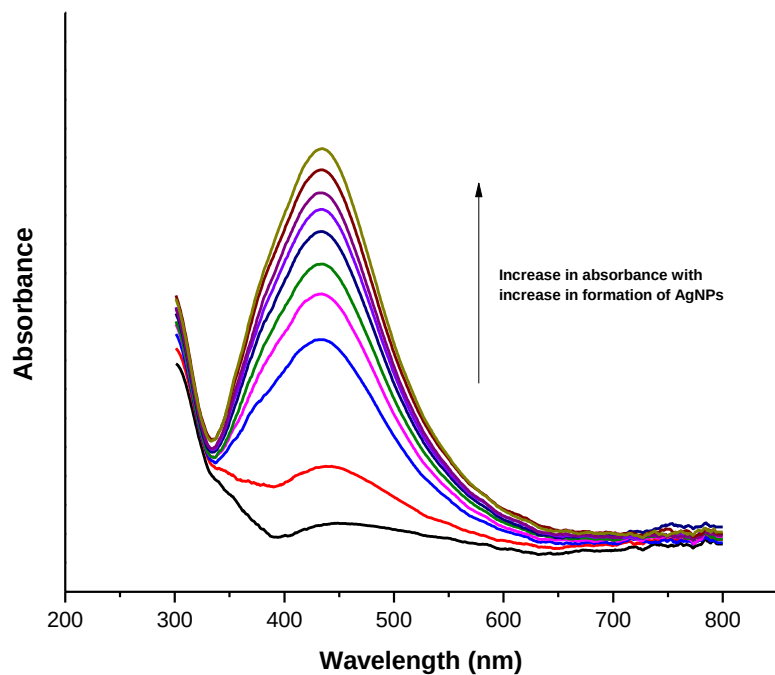


Figure 3.16: UV-Visible Spectra of the reaction mixture at 15 min time intervals over 3 h and a wavelength range of 200 to 800 nm

3.3.2.2 SEM Characterization of the Prepared AgNPs

Figure 3.17 is a SEM image showing spherical nanoparticles. The nanoparticles were brighter and easily identified by backscattered light in SEM images since metals like silver show higher secondary electron emission [300, 301].

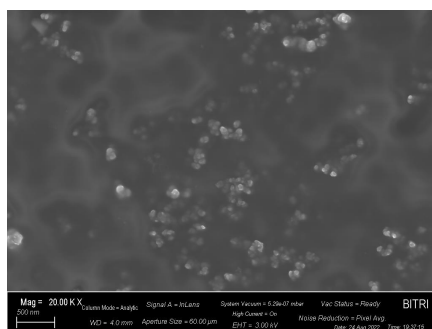


Figure 3.17: SEM micrograph of the biosynthesized AgNPs

3.3.2.3 Characterization of the Crystalline Structure of the Fabricated AgNPs Using XRD

Analysis through X-ray diffraction spectrophotometry was carried out to confirm the crystalline nature of the fabricated particles. The XRD pattern on **figure 3.18** depicts diffraction peaks at 37.54°, 46.94°, 66.68° and 77.90° which were indexed with planes (111), (200), (220) and (311) respectively, (JCPDS card no 04-0783, 1991). In the past, Gregory et. al. has [300] reported a diffraction peak at 32.20° and indexed it with the plane (101) in bio-synthesized AgNPs at room temperature. Likewise, we observed a peak at 32.19° which we have also indexed with the plane (101). On the other hand, the asterified peaks in **figure 3.18** were attributed to the existence of phytochemicals from the leaf extract and impurities in the matrix by Some et. al. and Palithya et. al. [302, 303]. The positions and relative intensities of the diffraction peaks matched well with those reported before for face-centered cubic structure of AgNPs by Kalaimurugan et. al. [304]. From this study, considering the peak positions and full width at half maximum, average particle size has been estimated by using Debye-Scherrer formula as shown on **equation 3.4** [305].

$$D = \frac{K\lambda}{\beta \cos\theta} \quad (3.4)$$

Where λ is the wavelength of the X-ray (Cu Ka = 0.1541 nm), β is full width at half maximum (FWHM) in radians, θ is the diffraction angle in radians, $K = 0.89$ is the Scherrer constant related to the shape and index (hkl) of the crystals and D is particle diameter size in nm. The average particle size was found to be about 33 nm.

3.3.2.4 Oxidase Mimetic Activity of the Bio-synthesized AgNPs Towards the Oxidation of TMB and its Inhibition by SCN⁻

The oxidase-like activity of the bio-synthesized AgNPs towards the oxidation of TMB by dissolved oxygen in the presence of AgNPs and in the presence and absence of SCN⁻ was monitored over time via UV-visible spectroscopy which showed differences in absorbance intensities at $\lambda_{\text{max}} = 654 \text{ nm}$ in the different reaction systems including; i. TMB +

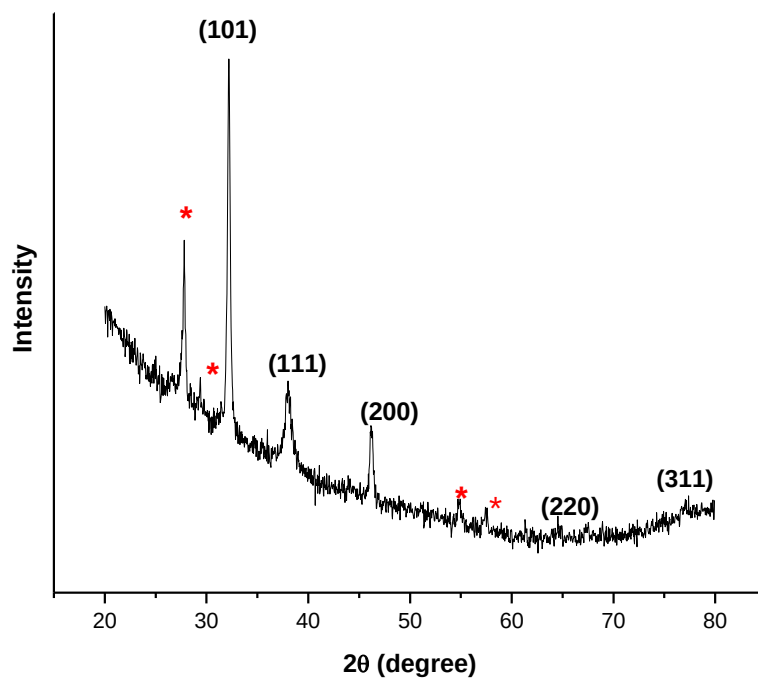


Figure 3.18: XRD spectrum of the bio-synthesized AgNPs

AgNPs, ii. TMB + AgNPs + SCN^- , iii. TMB alone and iv. TMB + SCN^- (see **figure 3.19**).

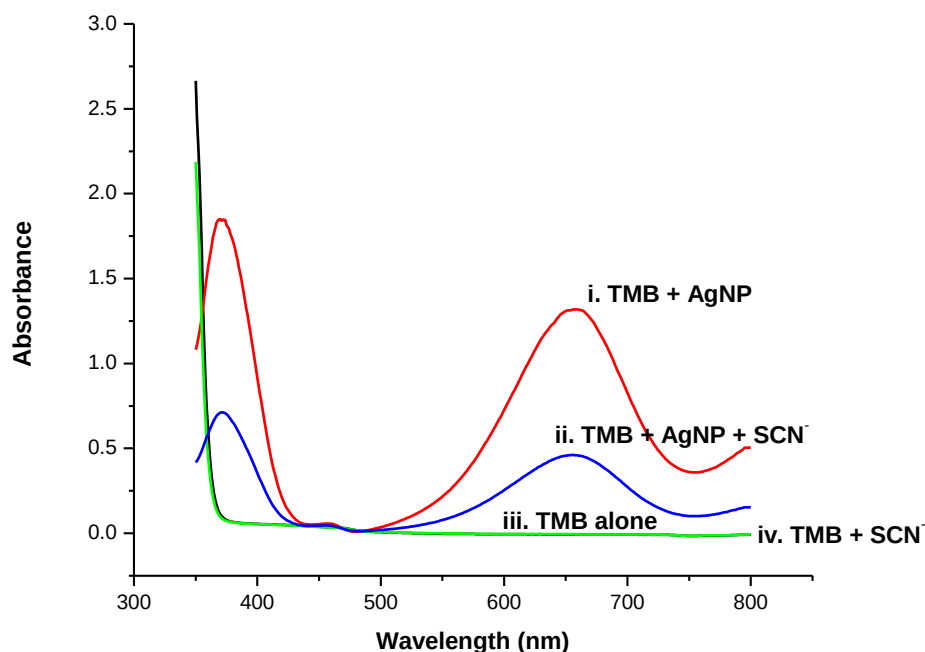


Figure 3.19: UV/Visible spectra of reaction systems containing TMB only, TMB and AgNP, TMB and SCN⁻ and TMB, AgNP and SCN⁻

Figure 3.19 i. spectrum shows the recorded absorbance for the AgNPs catalyzed oxidation of TMB by dissolved oxygen, with the appearance of the characteristic absorbance peak of the oxidized TMB (oxTMB) at 654 nm and a corresponding characteristic bright blue colored solution. According to Lee et. al. [306] the blue-colored product was caused by the one-electron oxidation of TMB in solution, producing a blue coloured charge-transfer complex of the parent compound and its imine oxidation product in equilibrium with its cation free-radical as shown on the reaction equation on **figure 3.20**.

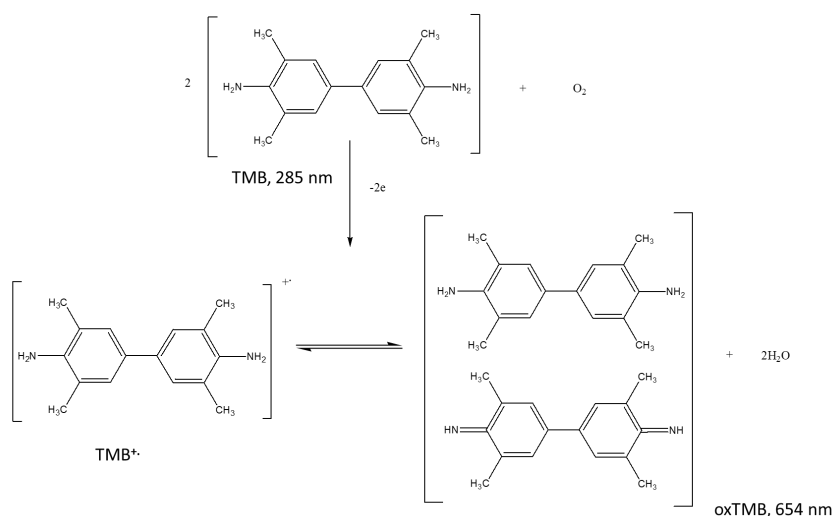


Figure 3.20: Oxidation of TMB by O_2

However, when $100 \mu\text{M SCN}^-$ was added to the system containing TMB and AgNPs, a light blue oxTMB was formed with the absorbance peak being reduced by a factor of about 3 when compared compared to the absorbance peak of i.TMB + AgNPs. This marked the inhibition of the catalytic activity of the AgNPs towards the oxidation of TMB by SCN^- . This observation has been explained before by Hu et.al. and Zhang et. al. who did similar work, to be due to SCN^- adsorbing onto the surface of the prepared metal nanoparticles resulting in the formation metal-S bonds [187, 188]. This has been said to block the active sites of such nanoparticles and hence inhibiting their catalytic property [243]. A schematic illustration for the effect of SCN^- on the catalytic oxidation of TMB which represents the main principle for this work is shown in **figure 3.21**. This principle was used to develop a AgNPs colorimetric probe for the screening of SCN^- via the catalytic oxidation of TMB in the presence of dissolved oxygen.

When TMB alone and TMB and SCN^- were monitored using UV-Visible spectroscopy, no peaks were observed at $\lambda_{\text{max}} = 654 \text{ nm}$ as can be seen by the flat absorbance graphs on **figure 3.19 iii. and iv.** for the two reaction mixtures. No color changes were also observed for these systems. This further confirmed that the suppressed catalytic oxidation of TMB was due to the interaction of SCN^- with the prepared AgNPs only, which acted as the catalyst.

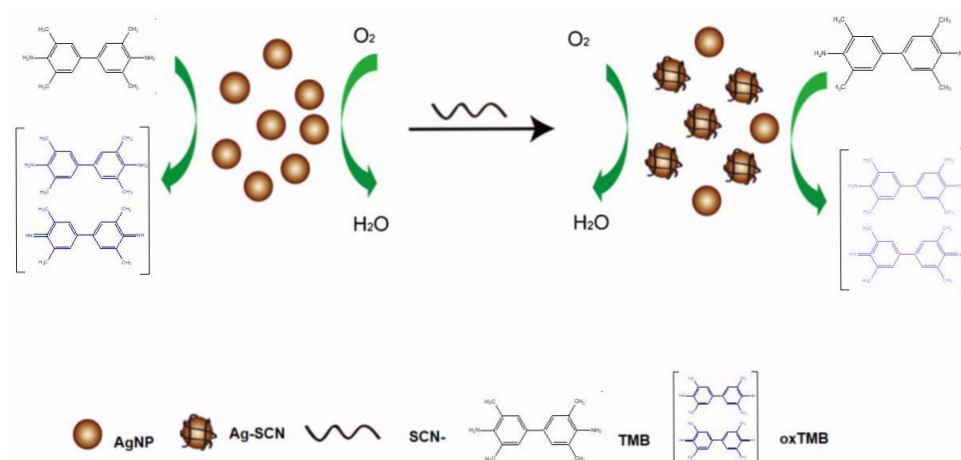


Figure 3.21: Schematic illustration of oxidase-like activity of AgNPs towards TMB and effect of SCN^- on the system

3.3.2.5 Optimization of Experimental Conditions

Like natural enzymes, the catalytic activity of the prepared AgNPs can be affected by concentration of the catalyst, pH, incubation time and concentration of the substrate, TMB. To obtain high sensitivity for SCN^- detection, these experimental conditions were optimized. The absorbance signal in the presence and absence of SCN^- , denoted A and A_0 respectively were used to calculate and plot the absorbance difference, ΔA where $\Delta A = A_0 - A$ against the different parameters.

- **Optimization of Volume of the Solution Containing the Prepared AgNPs**

The efficiency of the catalytic reaction relies on the quantity of catalyst available to catalyse the oxidation of TMB, hence the quantity of AgNPs was optimized. **Figure 3.22 a.** shows that the absorbance signal at $\lambda_{\text{max}} = 654 \text{ nm}$ increased with an increase in the quantity of AgNPs in the absence of SCN^- . This is because with increasing quantity of catalyst there were more active side available to absorb O_2 and TMB hence more oxTMB was formed. In the presence of SCN^- the absorbance intensities increased with increasing AgNPs although to a lesser extent compared to in its absence.

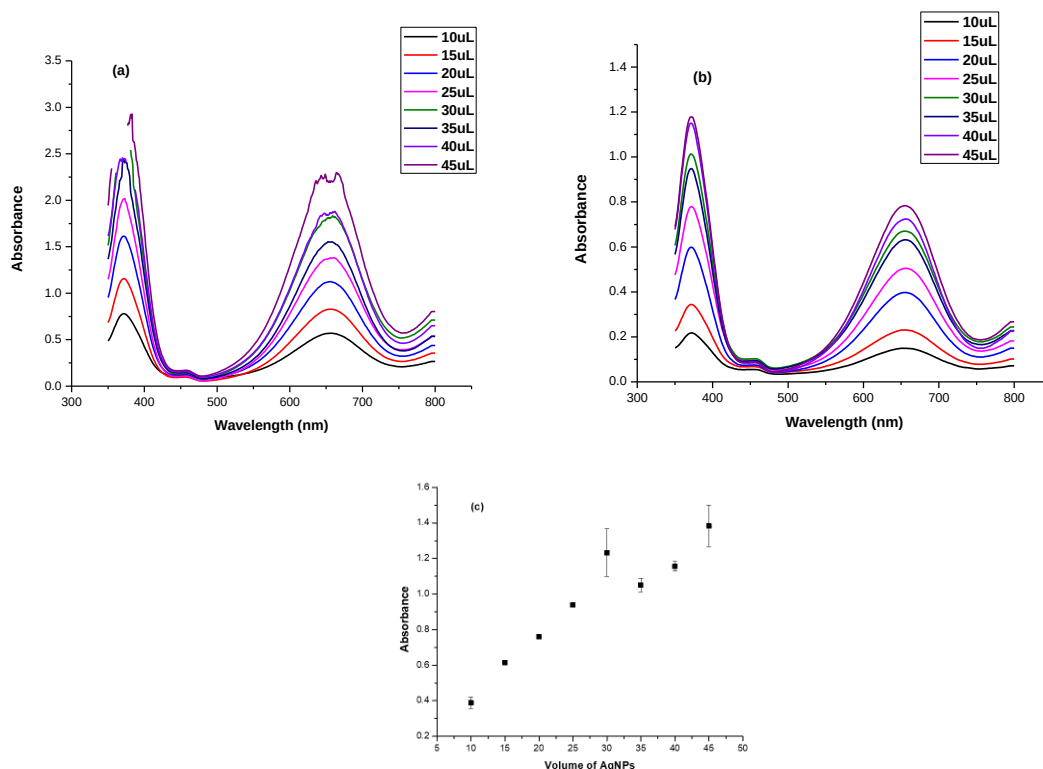


Figure 3.22: Effect of volume of solution containing AgNPs on the oxidation of TMB; spectra showing the effect of varying volumes of solution containing AgNPs on the oxidation of TMB a. in the absence and b. presence of SCN^- c. Plot of absorbance differences, ΔA in the presence and absence of SCN^- , $\Delta A = A_0 - A$ against the volume of solution containing AgNPs

This is evident on **figure 3.22 b.** and is because even though SCN^- blocked some of the active sites on the AgNPs, its effect was limited by excess AgNPs present in the system. The plot on **figure 3.22 c.** of the absorbance differences shows that the sensing system was more sensitive at 30 μL of AgNPs, marked by the highest point on the graph. Taking this into consideration 30 μL was selected as the optimal quantity of AgNPs. At this point on the graph, $A_0 - A$ was at its maximum.

- **Optimization of Concentration of TMB**

The influence of the concentration of TMB was investigated between concentrations 1.0-10.0 mM of TMB over the wavelength range of 300 - 800 nm as can be seen on **figure 3.23** spectra.

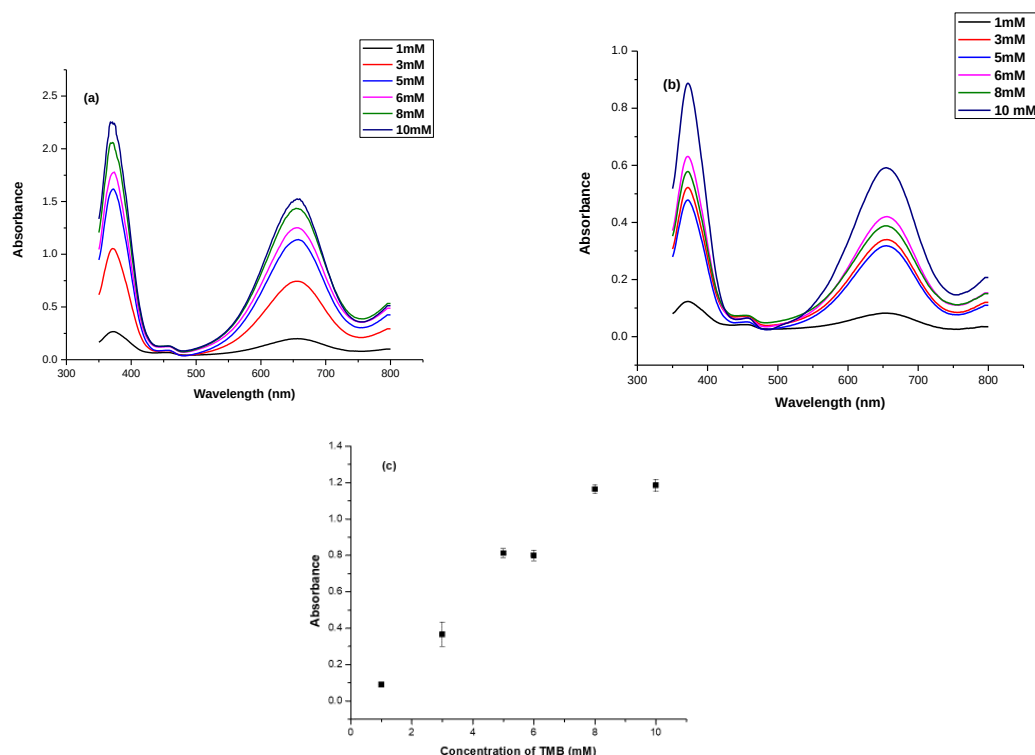


Figure 3.23: Effect of concentration of TMB on the reaction system; spectra showing the effect of concentration of TMB on the reaction system a. in the absence and b. presence of SCN^- c. Plot of absorbance differences, ΔA in the presence and absence of SCN^- , $\Delta A = A_0 - A$ against the concentration of TMB

On **figure 3.23 a.**, in the absence of SCN^- , the absorbance intensity at 654 nm increased with increasing TMB concentration. This meant that at each concentration level of TMB, there was more TMB available to be oxidised and the catalyst had enough active sites to accommodate the TMB molecules, hence more oxTMB was formed. On the other hand, in the presence of SCN^- , TMB was oxidised to a lesser extent even with increasing concentration of TMB. This is depicted by lower absorbance intensities at the same TMB concentration on **figure 3.23 b.** compared to **figure 3.23 a.** and it was due to the inhibition of active sites on the prepared nanozyme by SCN^- molecules. **Figure 3.23 c.** shows that the difference in absorbance intensities of oxTMB increased gradually with TMB concentration at lower levels until reaching a maximum at 8.0 mM then flattened. 8.0 mM was chosen as the optimal TMB concentration.

• Optimization of Incubation Time

Incubation time for the colorimetric screening of SCN^- via the catalytic oxidation of TMB was also assessed, in the presence and absence of SCN^- . As shown on **figure 3.24 a.**, in the absence of SCN^- the absorbance at 654 nm increased rapidly from 1 to 3 min signalling the oxidation of TMB with time. From then on, the absorbance only increased by a small margin. This shows that from the start of the experiment to 3 min there were a lot of active sites on the prepared nanozyme available for the adsorption of TMB and O_2 , however as the experiment progressed the active sites of the nanozyme became saturated such that the reaction rate was reduced hence the absorbances appear to be similar after 3 min.

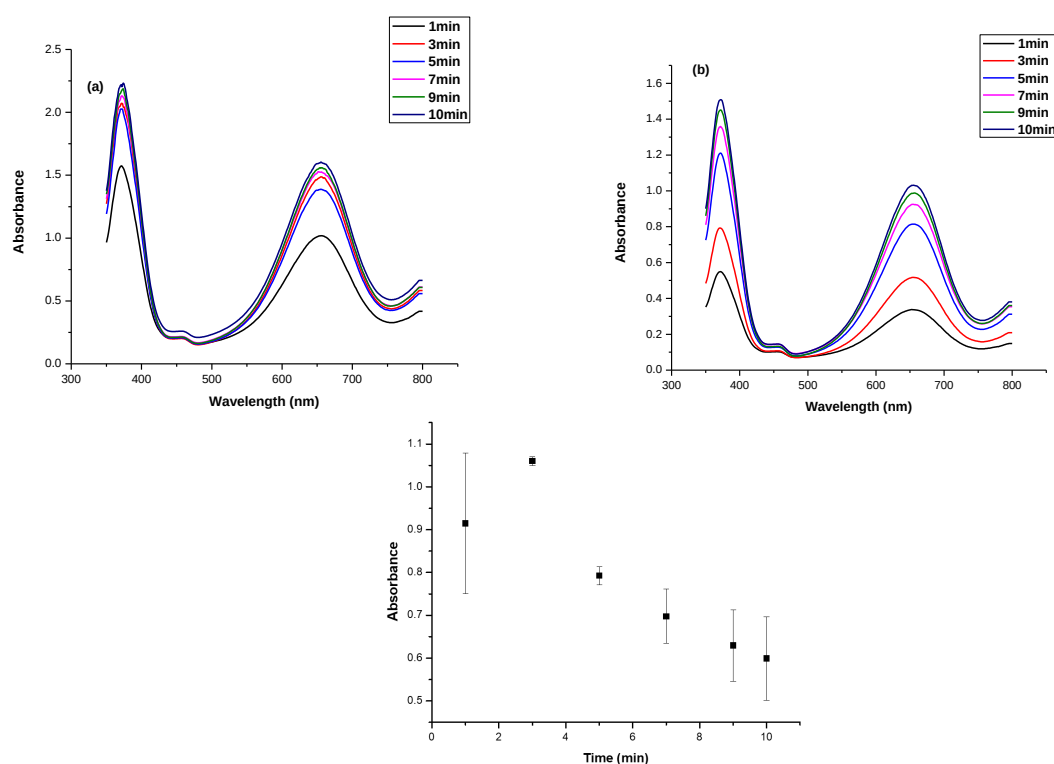


Figure 3.24: Effect of incubation time on the reaction system; spectra showing the effect of incubation time on the reaction system a. in the absence and b. presence of SCN^- c. Plot of absorbance differences, ΔA in the presence and absence of SCN^- , $\Delta A = A_0 - A$ against the incubation time

The spectrum on **Figure 3.24 b.** shows lower absorbance recorded for the same time intervals compared to **Figure 3.24 a.** This showed that in the presence of SCN^- reaction was slower,

this is because SCN^- inhibited the catalytic activity of the nanozyme. From the plot in **Figure 3.24 c.** the absorbance difference increased from 1-3 min and then decreased with increasing time. Therefore, for further experiments 3 min was used as the optimal incubation time.

- **Optimization of pH**

Like natural enzymes, the catalytic activity of AgNP-SCN^- system relied on pH. Therefore, the effect of pH on the catalytic activity of the nanozyme in the absence and presence of SCN^- was also studied. According to Drozd et. al. [307] the oxidation of TMB is acid-dependent due to its limited solubility in aqueous solutions, which is improved in acid media due to the protonation of one of amino groups, hence, in this work the optimization of pH was carried out in acidic media (pH values 3.0-6.0). As seen on the spectrum on **figure 3.25 a.** in the absence of SCN^- , at lower pH values the absorbance due to the oxidation of TMB was low. This is because at low pH values both the amino groups on the substrate are protonated, and this makes the substrate insusceptible to catalytic oxidation [307]. The absorbance for the oxidation of TMB was highest between pH values of 4.0 and 5.0. According to Alula et. al. for TMB, maximum oxidation reaction efficiency is usually observed in a pH range of 4.0 to 5.0 [243] due to protonation of one of the amino groups. Our results are in agreement with this. As shown in the spectrum on **figure 3.25 b.** the absorbance was lowest at pH 4 in the oxidation of TMB in the presence of SCN^- , this means that SCN^- was able to suppress the catalytic activity of the nanozyme more efficiently at pH 4. The graph on **figure 3.25 c.** shows that ΔA was highest at pH 4, therefore this pH was used as the optimal pH.

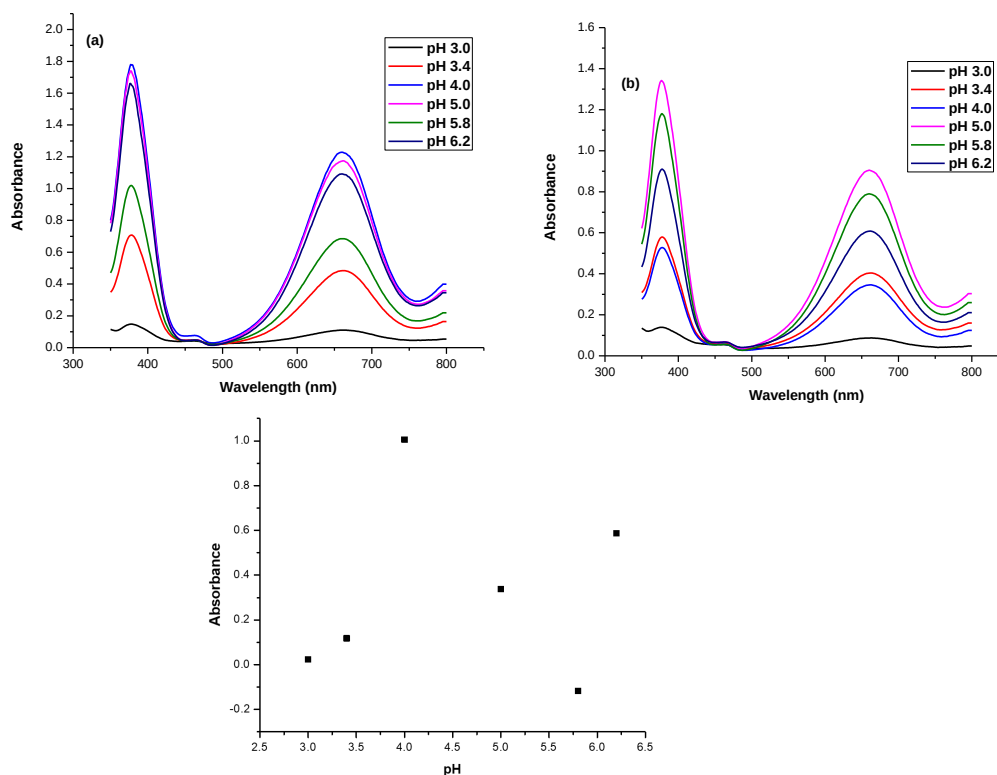


Figure 3.25: Effect of pH on the oxidation of TMB; spectra showing the effect of pH on the reaction system a. in the absence and b. presence of SCN^- c. Plot of absorbance differences, ΔA in the presence and absence of SCN^- , $\Delta A = A_0 - A$ against pH

3.3.2.6 Steady-State Kinetics

The steady-state kinetics for the catalytic activity of AgNPs towards the oxidation of TMB with dissolved oxygen were determined. The initial rate of catalytic reaction was estimated from Beer-lambert law.

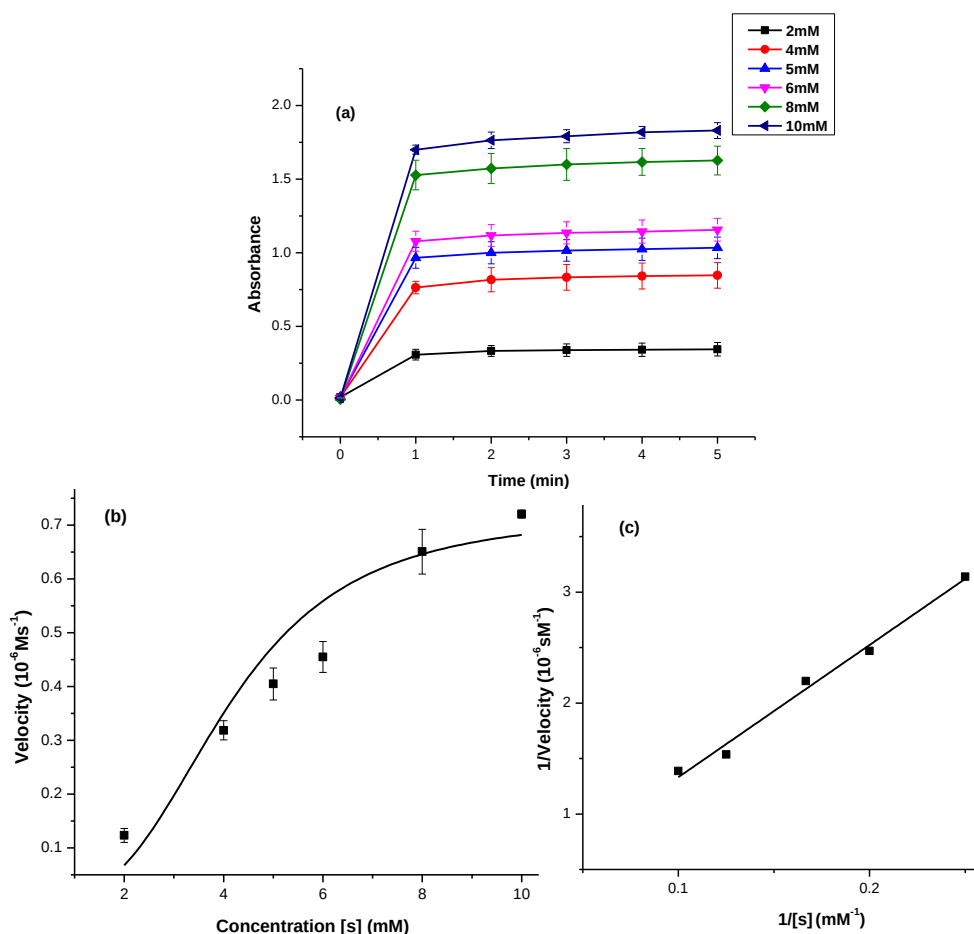


Figure 3.26: a. Steady state kinetics at varying TMB concentrations, b. Michaelis-Menten curve and c. the corresponding Lineweaver-Burk plot

Here the slope of absorbance at 654 nm per unit time in **figure 3.26 a.** was taken as the initial rate for a given range of concentrations of TMB. From these, a typical Michaelis–Menten curve was obtained as shown on **figure 3.26 b.** and the inverse of the Michaelis–Menten curve (Lineweaver-Burk plot) shown on **figure 3.26 c.** was plotted to obtain the maximal reaction velocity (V_{max}) and Michaelis–Menten constant (K_m). K_m was determined as 0.08461 mM which was rather low compared to the others in **table 3.3** [240, 242, 243]. The low K_m value reflected the high affinity of the nanozyme towards the substrate, TMB. We found V_{max} of 7.10 $\mu\text{M/s}$. This high V_{max} value meant that the nanozyme exhibited a high enzymatic activity towards TMB. K_m and V_{max} values of recently reported nanozymes for the detection of SCN^- and AgNPs nanozymes prepared in this study are given in **table 3.3**.

The lower K_m value of the prepared AgNPs nanozymes indicated stronger affinity of the AgNPs nanozymes towards TMB compared to the other nanozymes.

Catalyst	Substrate	k_m (mM)	V_{max} (M/s)	Reference
AgNP/FP	TMB	2.23	2.873×10^{-7}	[243]
Bimetallic PdCu	TMB	0.25	1.19×10^{-8}	[240]
Au QDs	TMB	0.38	-	[242]
AgNPs	TMB	0.085	7.10×10^{-6}	this work

Table 3.3: Michaelis–Menten constant, k_m and maximum reaction rate, v_{max} of AgNPs and other nanozymes used for the detection of SCN^- .

3.3.2.7 Efficacy of the Method

- **Linearity**

Linearity was determined by studying the effect of increasing the concentration of SCN^- on the absorbance intensities of oxTMB. **Figure 3.27 a.** shows UV-Vis spectra of TMB oxidation at different SCN^- concentrations. At 654 nm the absorbance decreased with increasing SCN^- concentration. The relationship between absorbance and concentration of SCN^- is presented on **figure 3.27 b.** Similarly, this shows a decrease in absorbance intensities of oxTMB with increase in concentration of SCN^- until about 80 μM when it plateaued. From this the calibration curve in the range 2-50 μM was obtained as shown on **figure 3.27 c.** The square of the correlation coefficient, $R^2 = 0.9944$ which is a measure of linearity was obtained. The value of R^2 obtained meant that for the studied concentration range there is a strong linear correlation between the concentration of SCN^- and the intensity. From this it can be concluded that the method was valid in the concentration range 2-50 μM for the detection of SCN^- . Compared to other colorimetric methods employing nanozymes for the detection of SCN^- (**table 3.4**) the current proposed method is not as sensitive. Nonetheless, the use of the plant extract in the fabrication of the nanozyme offers a more rapid, sustainable, non-toxic and eco-friendly option for the synthesis of nanozymes for the detection of SCN^- . Furthermore, the proposed silver nanoparticles nanozyme catalyzes the oxidation of TMB in the presence of oxygen as a green oxidant as opposed to the other nanozymes Which employ

unstable and hazardous chemical hydrogen peroxide as the oxidant. The method is also rapid compared to the others, which makes it a more ideal method for the onsite screening of SCN^- .

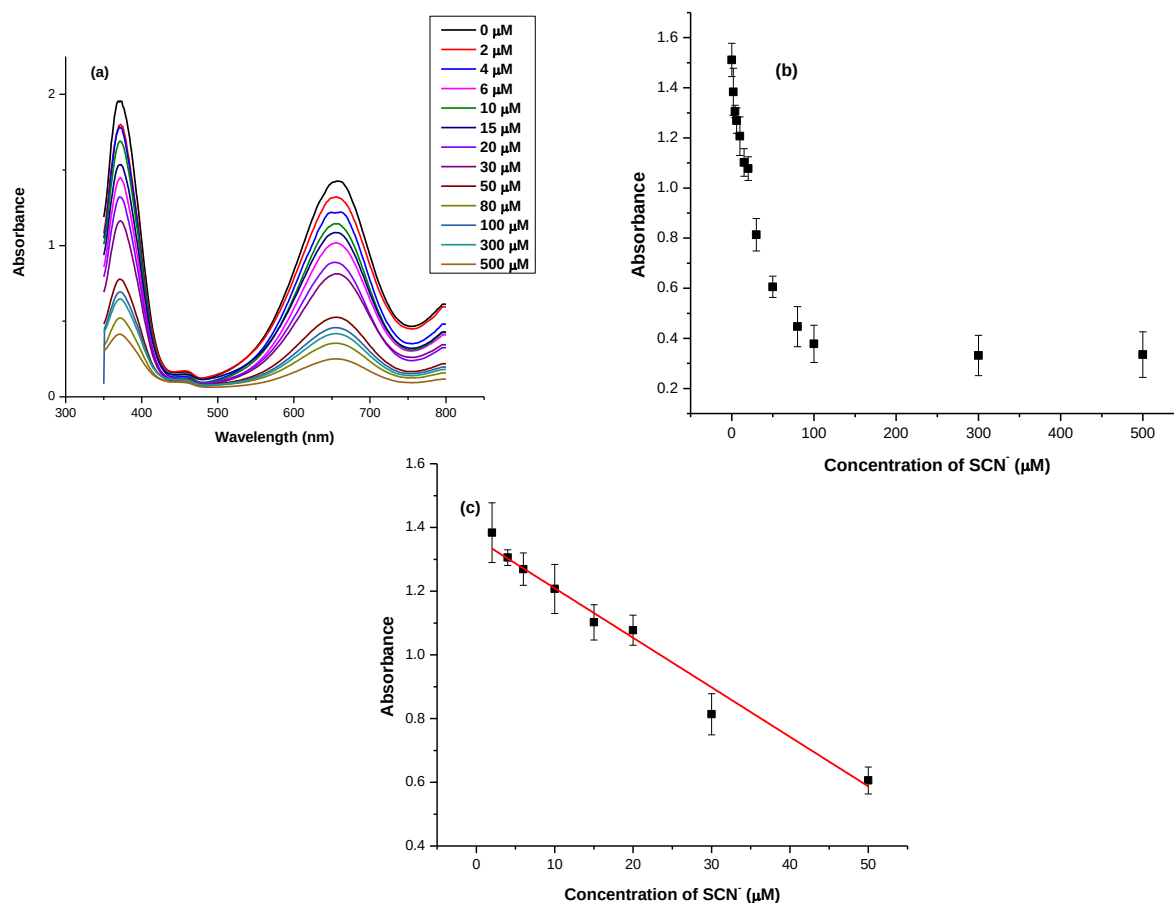


Figure 3.27: Sensitivity of AgNPs towards SCN^- . a. absorption spectra of TMB under varying concentrations of SCN^- (0-500 μM), b. plot of absorbance variation versus the concentration of SCN^- added and c. linear relationship between Absorbance and concentration of SCN^- (n=3)

Material	Linear range	Detection limit	reaction time (min)	Ref
Au@Pt NCs	20 nm - 40 μM	5 nM	10	[253]
GQDs-AuNPs	0-10 nM	3 nM	30	[241]
Au@ZnO/Fe ₃ O ₄	2-50 μM	2.89 μM	3	This worked
PdCu	0.001-100 μM	1 nM	5	[240]
AuQDs	2-100 nM	8 nM	10	[242]

Table 3.4: comparison of the proposed method with other reported Au-based nanozymes for the detection of Hg^{2+}

- **Limit of Detection LOD**

The LOD of the colorimetric sensor is calculated using the equation, $LOD = 3\sigma/k$, where k is the slope of the calibration curve, and σ is the standard deviation of the y-intercept. It was calculated to be 2.89 μM .

- **Precision and Recovery**

Sample	Spiked (μM)	Detected (μM)	Recovery(%)	% RSD
Milk	10.0	8.9	89.4	26.9
	15.0	17.7	118.1	23.6
	30.0	28.5	95.1	5.1

Table 3.5: Determination of SCN^- in real samples by UV-Vis

To assess the validity of the colorimetric method in detecting SCN^- in real samples, milk samples were spiked with different amounts of SCN^- . The results presented on **table 3.5** show that recoveries over the ranges 89.4-118.1 % and % RSD in the ranges 5.1-26.9 % for $n=3$ were obtained. These results indicated that the proposed method is reliable and suitable for practical application.

3.3.2.8 Effect of Potentially Interfering Compounds

To evaluate the selectivity of the assay, several potential interferents were studied, including anions (SO_4^- , Cl^- , NO_3^- , $\text{S}_2\text{O}_3^{2-}$), metal ions (K^+ , Ca^{2+} , Fe^{3+} , Mg^{3+} , Zn^{2+} , Cs^+), sugars (glucose, and fructose), amino acids (lysine, cysteine, glycine, and glutathione) and ascorbic acid, which may typically be present in milk. On **figure 3.28 a.** the SCN^- had the lowest absorbance intensity compared to potential interferants, this means that it has the great inhibitory effect on the catalytic activity of the prepared AgNPs nanozymes. Of the possible interferents analyzed only glutathione (which also has a thiol functional group) seemed to considerably inhibit the catalytic activity of AgNPs although to a lesser extent compared to SCN^- . When SCN^- was analyzed in the presence of competing species **Figure 3.28 b.**, its

inhibitory effect was maintained although to a lesser extent compared to when SCN^- was analyzed alone. These results have been analyzed on the bar graph on **figure 3.28 c.** The selective interaction of SCN^- with the AgNPs is due to SCN^- adsorbing onto the surface of the prepared AgNPs resulting in the formation Ag-S bonds [187, 188]. This has been said to block the active sites of the nanoparticles and hence inhibiting their catalytic property [243].

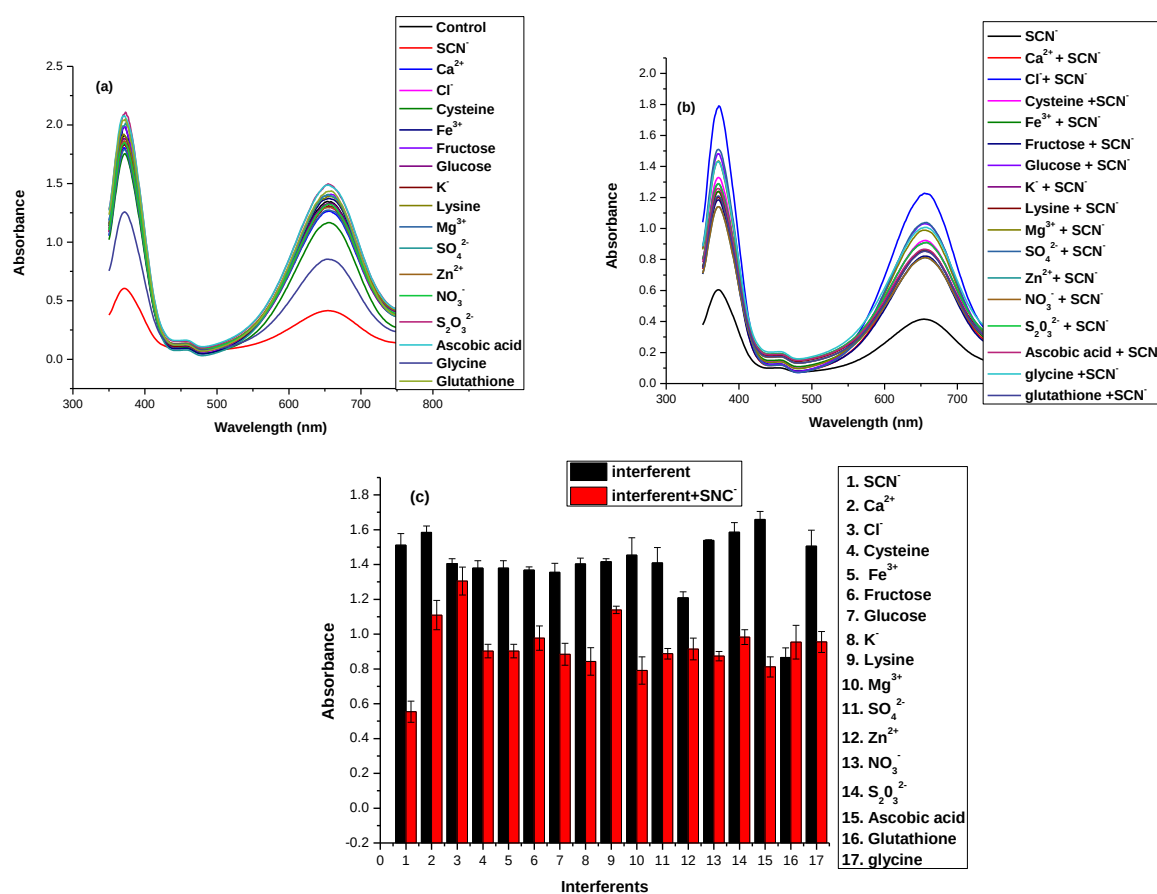


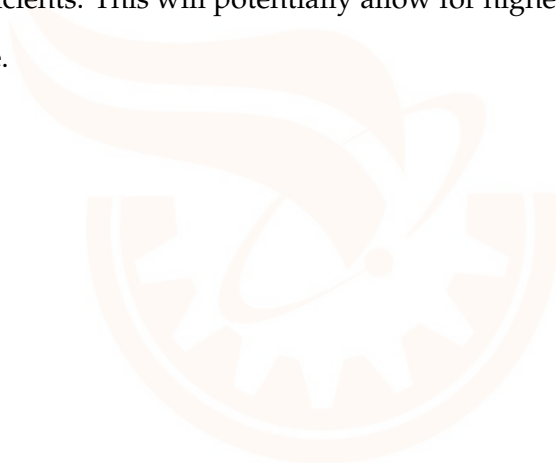
Figure 3.28: Selectivity of the AgNPs with oxidase-like property for determining SCN^- . a. UV-vis spectra of systems containing TMB and potentially interfering species, b. UV-vis spectra of systems containing TMB and SCN^- in the presence of potentially interfering species c. bar graph showing absorbance intensities of the reaction solution in the presence of potentially interfering species

Chapter 4

Conclusions and Future Work

The work presented in this thesis outlines the potential and applicability of novel catalytic nanoparticles with enzyme mimetic characteristics known as nanozymes that were fashioned into simple, rapid, cheap, sensitive and selective colorimetric probes for the screening of restricted substances commonly added to consumer products illegally as substitutes for legitimate ingredients. The final products were two novel colorimetric probes that were able to screen for Hg^{2+} and SCN^- as model restricted substances at different concentrations from 0 to 10 nM and 2 to 50 μM , respectively, based on gradation in color and absorbance. These concentration ranges allowed for the trace analysis of Hg^{2+} and SCN^- in consumer products. Both probes exhibited very low limits of detection (LoDs); 2.34 nM for the $\text{Au@ZnO/Fe}_3\text{O}_4$ probe and 2.89 μM for the AgNPs probe which were well below acceptable levels of 4.99 μM for mercury in cosmetics and 0.24 mM for thiocyanate in milk as set by the US Food and Drug Administration (FDA) and International Dairy Federation (IDF), respectively. The practical applicability of using these colorimetric probes for Hg^{2+} and SCN^- detection in complex matrices such as a skin lightening cosmetic product and milk, respectively was also assessed. From the studies, high recoveries ranging from 89.4 to 118.1 % and high precision marked by low % RSDs of 4.3-26.9 % for $n=3$ for both probes were also obtained. Excellent selectivities, toward Hg^{2+} and SCN^- owing to the specific interactions between the prepared nanozymes and the adulterants over potentially interfering species were also achieved.

The work discussed in this thesis offers great strides in strategies for the analysis of adulterants in consumer products that may influence consumer awareness education and policy formulation in monitoring of restricted chemicals. However, more research needs to be carried out in optimizing the synthetic methods for the presented nanozymes. Such work will contribute towards improving their catalytic activities and understanding their catalytic mechanisms. Additionally, in this work, only the reductase and oxidase-like properties of the prepared nanoparticles were explored. It is important to assess the materials further for other enzyme-mimetic activity that could potentially be applied in other fields of analysis. Furthermore, only TMB and 4-NP were applied as chromogenic substrates. While these are convenient and useful, it is also important to explore other potential substrates with higher extinction coefficients. This will potentially allow for higher sensitivity thus improving probe performance.



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