DEVELOPMENT OF COLORIMETRIC AND LIGHT SCATTERING METHODS FOR DETERMINATION OF GAMMA-AMINOBUTYRIC ACID IN FOODS USING SILVER NANOPARTICLE SENSOR

AMORNRASSAMEE JINNARAK

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENT FOR THE DEGREE OF PHILOSOPHY IN APPLIED CHEMISTRY
DEPARTMENT OF CHEMISTRY, FACULTY OF SCIENCE
KING MONGKUT'S INSTITUTE OF TECHNOLOGY LADKRABANG
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Thesis Title	Development of Colorimetric and Light Scattering Methods for Determination of Gamma-aminobutyric acid in Foods Using Silver Nanoparticle Sensor
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Student ID	55650307
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Thesis Advisor	Assistant Professor Dr.Saowapak Teerasong

Abstract

A development of two detection methods for determining Gamma-aminobutyric acid (GABA) in foods based on silver nanoparticles (AgNPs) was presented. The reaction between AgNPs and GABA was based on electrostatic interaction. AgNPs were stabilized with negatively-charged citrate ions. At an acidic pH of 3.8, GABA had a positive charge due to protonation of its amine group. When GABA was introduced into AgNP suspension, positively-charged GABA neutralized the negatively charged citrate on the surface of AgNPs. This resulted in aggregation of particles. The aggregation caused changes in AgNPs absorption and scattering properties that could be detected by spectrophotometry. The first method was a GABA detection method based on light absorption while the second involved detection based on light scattering. An assembled sequential injection (SI) system was also used in the light scattering method in order to automate detection operations.

The aggregation caused a decrease in the surface plasmon resonance (SPR) spectra of the AgNPs suspension at the wavelength of 390 nm with a slight red shift. At the same time, the color of the suspension turned from yellow to green. The quantification of GABA was done based on the spectrophotometrically measured SPR absorbance at this wavelength. Under optimum conditions, the method yielded a linear calibration range of 100–500 mg L^{-1} GABA with a detection limit of 57.7 mg L^{-1}.

The method was successfully applied to measurement of the GABA level in food supplement samples.

Intensity of scattered light can be detected by a spectrofluorometer. In the second work, a sequential injection with second order light scattering (SOS) was developed for GABA analysis. Working standard solutions of GABA were prepared in-line by the SI system pumping appropriate volumes of a stock solution of GABA and acetate buffer into a holding coil. The suspension of AgNPs was subsequently drawn into the coil. The reaction zone was then transferred into the spectrofluorometer, set with excitation and detection wavelengths at 300 and 600 nm, respectively. The SOS intensity was proportional to the concentration of GABA. Under optimal conditions, a linear calibration curve was obtained in the range of 100– 400 mg L^{-1} GABA with a limit of detection of 39.6 mg L^{-1}. The developed method was successfully applied for quantification of GABA in food supplement and instant green tea samples.

Keywords: Gamma-aminobutyric acid, absorption, light scattering, sequential injection analysis, silver nanoparticles

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Chapter 1
Introduction

1.1 Research motivation

Gamma-amino butyric acid (GABA) is a neuroactive amino acid that is produced in mammalian brains by decarboxylation of l-glutamate catalyzed by glutamate decarboxylase. An inhibitory neurotransmitter, GABA is widely distributed throughout the brain and found in 30–40% of all synapses. GABA is known as a natural calming agent: it inhibits over-stimulation of the brain, helps promote relaxation and eases nervous tension. The calming effect of GABA has been demonstrated in a study showing that GABA in a variety of tea products was able to reduce blood pressure in spontaneously hypertensive rats [1] and also in another study showing that it promoted sleep [2]. Because of this beneficial property, GABA has been added to many formulations of food supplement products. In order to include the exact specified amount of GABA as stated in the product label, the GABA content in the product has to be determined accurately.

Nanoparticles have attracted a lot of attention in fields such as chemistry, physics, medicine and photonics due to their distinctive physical and chemical properties. The optical properties of noble metal nanoparticles have fascinated many scientists in the recent past because of an interesting phenomenon known as surface plasmon resonance (SPR). SPR is an optical phenomenon that occurs when light wave gets trapped in conductive NPs smaller than its wavelength and the frequency of the incident photon is in resonant with the collective oscillation of conduction electrons in the metal NPs. This interaction produces coherent plasmon oscillation with resonant frequencies that strongly depend on the composition, size, geometry, dielectric environment and particle–particle separation distance. The interaction of NPs with light allows some photons to be absorbed and some to be scattered. The selective photon absorption and scattering allow convenient monitoring of SPR properties of NPs: surface plasmon resonance absorption (SPR-A) and surface plasmon resonance light scattering (SPR-LS). Both resonance absorption and scattering properties have been used for determination of various chemical species [3].

Common materials that are used for NP production are noble metals such as Ag and Au, which due to d–d transition energy levels, exhibit SPR in the visible range of the electromagnetic spectrum. Although both of them exhibit excellent optical properties and have attracted a lot of interest from researchers, AgNPs has certain advantages over AuNPs—higher extinction coefficient than that of AuNPs of the same size and much lower preparation cost [4].

In this work, two GABA detection methods based on AgNPs were developed. The study was divided into two parts. The first part involved using AgNPs as a colorimetric sensor for detecting GABA. AgNPs were prepared by reducing $AgNO_3$ with $NaBH_4$ and the produced nanoparticles were stabilized with sodium citrate dehydrate. The surface of citrate-stabilized AgNPs is electronegatively charged, hence when they are suspended in water, AgNPs are well dispersed due to electrostatic repulsion. GABA was prepared in a form of positively charged species by dissolving this compound in acetate buffer pH 3.8 of which the pH value was lower than the isoelectric point of GABA. When positively charged GABA were introduced into AgNPs suspension, the NPs were induced to aggregate. The color of the suspension changed from yellow to green. This phenomenon allowed the use of colorimetric AgNPs method to determine the amount of GABA.

The second part involved a development of GABA determination method based on light scattering phenomenon and automated by sequential injection analysis (SIA). Aggregation of AgNPs occured when it interacted with GABA. The interaction led to an increase in the size of AgNPs with an increase in GABA concentration. SIA has been proposed due to various advantages such as fast operation, high precision and consuming less reagents. Moreover, SIA makes it possible to perform continuous detection of GABA. Both developed methods were then applied to detect GABA in food samples.

1.2 Objectives of the study

Part 1

1.2.1 To synthesize AgNPs by using sodium borohydride as reducing agent and sodium citrate as stabilizing agent

1.2.2 To investigate the interaction between GABA and $AgNP_S$

1.2.3 To develop a simple method for detecting GABA based on surface plasmon resonance absorption

1.2.4 To find the optimal conditions for GABA analysis

1.2.5 To apply the developed method for detection of GABA in food supplement samples

Part 2

1.2.6 To develop an automated SIA method for detection of GABA based on light scattering measurement

1.2.7 To find the optimal conditions for the SIA method

1.2.8 To apply the developed method for detection of GABA in food samples

1.3 Scope of the study

Part 1

AgNPs were prepared by using sodium borohydride as reducing agent and sodium citrate as stabilizing agent.

⇩

The optical properties and morphology of AgNPs were characterized by UV-visible spectrophotometry, transmission electron microscopy (TEM) and zeta potential analysis.

⇩

The electrostatic interaction between GABA and AgNPs was investigated.

⇩

Parameters affecting the interaction of GABA and AgNPs such as stabilizer concentration, surrounding medium, pH value, acetate buffer concentration, concentration of AgNPs and reaction time were optimized.

⇩

Analytical performances of the method were determined.

⇩

The method was applied to detection of GABA in food supplement samples.

⇩

The results obtained by the developed method were validated against the results obtained from a spectrophotometric method.

Part 2

An automated SIA system with light scattering detection was constructed for GABA detection.

⇩

Flow parameters affecting the sensitivity of detection such as sequence zone, flow rate, mixing coil length, acetate buffer concentration, AgNPs concentration and AgNPs volume were optimized.

⇩

Analytical performances of the SIA method were determined.

⇩

The SIA method was applied to detection of GABA in food samples.

⇩

The results obtained by SIA method were validated against the results obtained by an HPLC method.

1.4 Benefits of the study

Two methods based on the use of optical properties of AgNPs for determination of GABA were achieved. The methods could be advantageously applied to routine analysis of GABA in food samples.

Chapter 2
Theory and Literature Reviews

2.1 Gamma-aminobutyric acid

Gamma-aminobutyric acid (GABA) is a major neurotransmitter widely distributed throughout the central nervous system (CNS). GABA is primarily formed by an irreversible α-decarboxylation reaction of L-glutamic acid or its salts, catalyzed by glutamic acid decarboxylase enzyme (GAD; EC 4.1.1.15) and its cofactor pyridoxal 5' phosphate (P5P; active vitamin B_6). (Figure 2.1) [5]. GABA plays an important role as an inhibitory neurotransmitter. It functions at neuron interconnection, inhibiting signal transmission and putting the neural activity into equilibrium hence helping the brain and mind to relax and get to sleep. It also stimulates the anterior pituitary gland which produces human growth hormone that stimulates tissue and muscle generation as well as a lipotropic substance that prevents accumulation of fat in the body

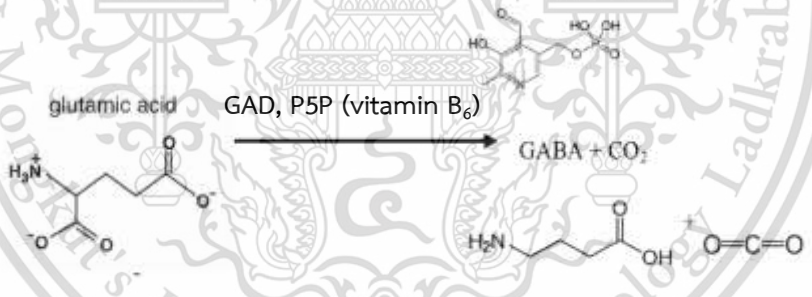


Figure 2.1 Decarboxylation reaction of L-glutamate to GABA catalysed by glutamate decarboxylase (GAD), which is dependent on the cofactor pyridoxal-5′-phosphate or vitamin B_6 [5].

2.1.1 Sources of GABA [6]

The body gets GABA from 2 sources.

2.1.1.1 External food

GABA is naturally present in small quantities in many plant sources, i.e., in vegetables such as spinach, potatoes, cabbage, asparagus, broccoli and tomatoes, in fruits, such as apples and grapes, and in cereals, for example barley

and/or maize. Recently, it has been suggested that germinated millets and legumes are a good source of GABA. A high amount of GABA is found mainly in fermented products, especially fermented dairy products, soy sauces and cheeses.

2.1.1.2 Internal synthesis within the body

GABA is synthesized in the body. The synthesis process starts from glycolysis of glucose into pyruvate. The pyruvate then enters into Krebs cycle turning into α-ketoglutarate that is in turn turned into glutamic acid, a precursor of GABA. Glutamic acid is produced from α-oxoglutarate in the Krebs cycle by the action of α-oxoglutarate transaminase (GABA-T) as shown in Figure 2.2, then it is turned into GABA by an irreversible α-decarboxylation reaction catalyzed by GAD and pyridoxal 5' phosphate (P5P; active vitamin B_6). A high level of synthesized GABA is found in the brain. Therefore, as the body ingests glucose, the brain will synthesize GABA and make the body feel relaxed. Although, human body can produce its own supply of GABA, but GABA production is sometimes inhibited by lack of estrogen, zinc or vitamins, or by an excess of salicylic acid and food additives.

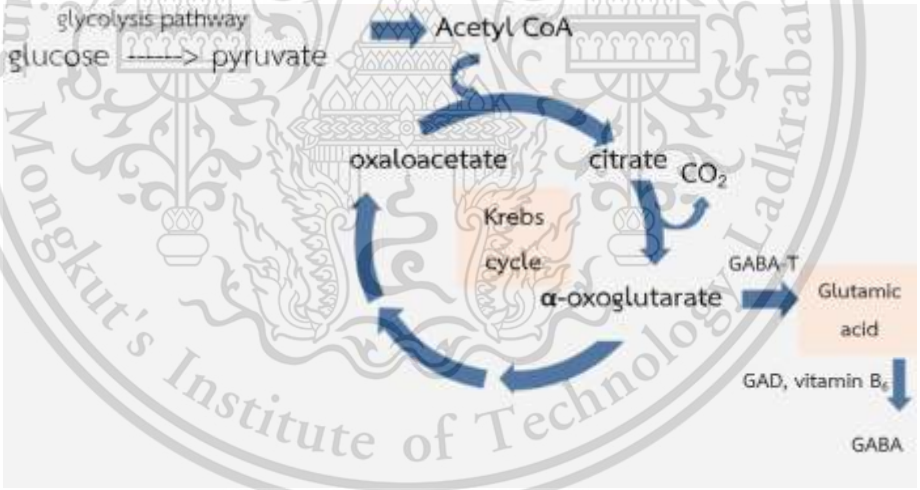


Figure 2.2 GABA synthesis from glucose via an intermediate in Kreb's cycle by the action of GABA α-oxoglutarate transaminase (GABA-T) [6].

2.1.2 GABA benefits to health [6]

At present, GABA is widely known. People are interested in consuming foods that are high in GABA because of many research reports on its beneficial effects to health. Among them are the benefits of reducing the risk of or even retarding the symptoms of many diseases which is described below.

2.1.2.1 lowering high blood pressure

Inoue et al.[7] attempted to help treated patients who were mildly hypertensive by letting them take yoghurt that contained high GABA and found that their blood pressure was reduced significantly within 2-4 weeks of intake. The reduction went on up to 12 weeks as the patients continued to take the yoghurt. The average reduction of systolic and diastolic blood pressures were 17.4+/-4.3 mmHg and 7.2+/-5.7 mmHg, respectively. The results of this kind of reduction were less headache from high blood pressure, fewer complications from high blood pressure such as heart diseases, myocardial ischemia, cardiomegaly, kidney disease, and protein leak into urine.

2.1.2.2 Preventing the spread of cancer cells

Oh et al [8] injected a GABA extract into cells of mouse leukemia L1210, cells of human acute lymphoblastic leukemia Molt4, and human cervical cancer HeLa cells. Compared the results from the control, GABA was found to be able to prevent these cancer cells from spreading. Moreover, it also helped producing apoptosis of cells of Mouse leukemia L1210. This pharmacological effect of GABA is being further developed.

2.1.2.3 Benefit to treatment of chronic alcoholism

Oh et al [9] investigated the levels of lipoprotein and cholesterol in the serum of the liver as well as aspartate aminotransferase and alanine aminotransferase enzymes that are indicators of the severity of liver destruction. The investigation was done in rats that were fed with food that contained ethanol and with food that contained ethanol and GABA with the control as food containing none of these substances for 30 days. It was found that the rats ingesting food with ethanol had a high level of low-density lipoprotein cholesterol (LDL-C) in the serum that is the cause of atherosclerosis as well as higher levels of aspartate aminotransferase and alanine aminotransferase. On the other hand, the rats that ingested food with ethanol and GABA had a higher level of high-density lipoprotein cholesterol (HDL-C) which is a good cholesterol in the serum and lower levels of enzymes that destroy liver cells. Moreover, it was found that GABA intake also prevent higher triglyceride and total cholesterol levels from ethanol intake. To conclude, GABA can be used as a nutrient treatment and prevention of diseases related to alcoholism.

2.1.2.4 Benefit to memory and learning

Ito and Ishikawa [10] found that GABA can reduce the occurrences of Alzheimeric symptoms, forgetfulness, and anxiety. Later on, Mamiya et al. (2007) presented a paper supporting the results from Ito and Ishikawa's study regarding the effect of GABA on learning that would help reduce Alzheimeric symptoms. They performed an experiment with rats and found that consumption of germinated brown rice that contained 15 times higher GABA than brown rice was able to help prevent brain cell destruction from Beta-amyloid peptide that is the cause of Alzheimer's disease.

2.1.2.5 Benefit of alleviating stress

Abdou et al [11] reported that their experimental results indicate that GABA, glutamine, and glycine intakes can reduce stress, anxiety, and forgetfulness. The mechanism of GABA starts from $GABA_A$ receptors bond with GABA causing more Cl^- intake into the cells as shown in Figure 2.3 making the intracellular fluid more negatively charged which is a condition that makes it difficult for neurons to be stimulated hence they are more at rest. Therefore, GABA has been brought in to treat anxiety, insomnia, and epilepsy.

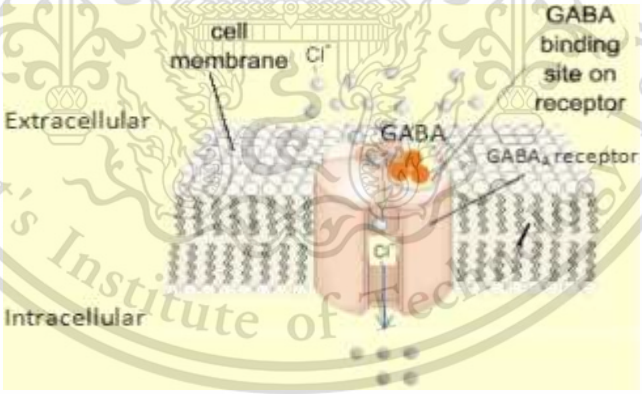


Figure 2.3 Mechanism of chloride ion intake into cells mediated by GABA [6].

2.1.3 Side effects and toxicity of GABA [12]

Although synthetic GABA-agonist drugs can have significant side effects, ranging from drowsiness and dizziness to addiction, natural GABA supplementation is virtually without side effects. This difference in safety profiles may result from a limited capacity of the brain to retain an excessive amount of GABA, since there is an efficient

efflux of GABA across the blood-brain barrier. LD_{50} tests conducted on natural GABA at doses of 5,000 mg Kg^{-1} to rats did not cause any mortality, indicating an LD_{50} >5,000 mg Kg^{-1} in rats.

2.1.4 Normal dosage [12]

A typical dosage of GABA for anxiety or sleep disorders is 100-200 mg up to three times daily. Dosages of GABA that were found to benefit a subset of individuals with epilepsy ranged from 1,500-2,500 mg daily.

2.1.5 Warnings and contraindications [12]

GABA has not been tested for safety in pregnant females. Due to its effect on neurotransmitters, it is not recommended during pregnancy or lactation.

2.2 Nanoparticles [13]

Nanoscience and nanotechnology are recent revolutionary developments of science and technology that are evolving at a very fast pace. They penetrate all areas of physical and chemical sciences, biological sciences, health sciences, and other interdisciplinary fields. Particles with sizes in the range of 1–100 nm are called nanoparticles (NPs). NPs are a number of atoms or molecules bonded together and are intermediate in size between individual atoms and aggregates large enough to be called bulk material. NPs are divided into metallic, non-metallic, semiconductor and organic NPs. NPs have high surface to volume ratio and are influenced by quantum-size effect and electro-dynamic interactions. Metallic NPs, good representatives of NPs, possess unique optical, electronic, chemical, and magnetic properties that are strikingly different from those of the individual atoms and their bulk counterparts. Both gold and silver, ancient noble metals, have NPs that show novel colors and have received considerable attention from researchers. The intense absorption and scattering of light from noble-metal NPs are the source of the beautiful colors in stained glass windows and have attracted the interest of many scientists. Although scientists have known that the characteristic hues of these noble-metal-NP suspensions arise from their strong interaction with light, the advent of NP optics has allowed a deeper understanding of the relationship between material properties (e.g., composition, size, shape, and local dielectric environment) and the observed color of a metal suspension.

An understanding of the optical properties of noble-metal NPs, especially gold and silver, is of both fundamental and practical significance.

2.2.1 Surface plasmon resonance of AgNPs [13]

Free electrons in the metal (d electrons in silver and gold) travel through the material. The mean free path in gold and silver bulk is ~50 nm. In particles smaller than ~50nm, no scattering is expected from the bulk. This means interactions with the surface dominate. When the wavelength of light is much larger than the nanoparticle size it sets up standing resonance conditions as shown in Figure 2.4. Light in resonance with the surface plasmon oscillation causes the free-electrons in the metal to oscillate. As the wave front of the light passes, the electron density in the particle is polarized to one surface and oscillates in resonance with the light's frequency causing a standing oscillation. The resonance condition is determined from absorption and scattering spectroscopy and is found to depend on the shape, size, and dielectric constants of both the metal and the surrounding material. This is referred to as the surface plasmon resonance (SPR), since it is located at the surface. As the shape or size of the nanoparticle changes, the surface geometry changes, causing a shift in the electric field density on the surface. This causes a change in the oscillation frequency of the electrons, generating different cross-sections for the optical properties including absorption and scattering.

Figure 2.4 Origin of surface plasmon resonance due to coherent interaction of the electrons in the conduction band with electromagnetic field [13].

The interaction of the metal nanoparticles can be described by Maxwell's equations and was solved for the case of spherical nanoparticles by Mie. The total

extinction and scattering efficiency Q_{ext} and Q_{sca} for a homogenous sphere are expressed in the following infinite series:

$$Q_{ext} = \frac{2}{x^2}\sum_{n=1}^{\infty}(2\varepsilon+1)\mathrm{Re}\left[a_n + b_n\right] \quad (2.1)$$

$$Q_{sca} = \frac{2}{x^2}\sum_{n=1}^{\infty}(2\varepsilon+1)\left[a_n^2 + b_n^2\right] \quad (2.2)$$

$$Q_{abs} = Q_{ext} - Q_{sca} \quad (2.3)$$

where Re is real part of; ε is the refractive index of the metal; ε_m is the refractive index of the surrounding medium; m is the ratio of the refractive index of the sphere and the surrounding medium ($m = \varepsilon/\varepsilon_m$); R is the radius of the sphere; λ is the wavelength of the light; and x is the size parameter ($x = 2\pi n_m R/\lambda$). These expressions have been solved in the dipole approximation for spherical nanoparticles much smaller than the wavelength of light (<20nm) where only the dipole contributes to the absorption by the nanoparticle,

$$Q_{abs} = \frac{18\pi V \varepsilon_m^{3/2}}{\lambda} \times \frac{\varepsilon_2(\lambda)}{\left[\varepsilon_1(\lambda) + 2\varepsilon_m\right]^2 + \varepsilon_2(\lambda)^2} \quad (2.4)$$

where V is the volume of the nanoparticle; ε_m is the dielectric constant of the surrounding medium; λ is the wavelength of the light; and the dielectric constant of the metal is expressed in a complex form as a function of the wavelength of light, where $\varepsilon(\lambda) = \varepsilon_1(\lambda) + i\varepsilon_2(\lambda)$. This allows the calculation of the expected absorption and scattering spectra of small spherical metal nanoparticles. Changing the dielectric constant of the surrounding material has an effect on the oscillation frequency due to the varying ability of the surface to accommodate electron charge density from the nanoparticles. The expected shift in the absorption spectrum with changing medium dielectric constant (ε_m) can be calculated using equation 2.4. Changing the solvent changes the dielectric constant, but the capping material is the most important in determining the shift of the plasmon resonance due to the local nature of its effect on the surface of the nanoparticle. Chemically-bonded molecules can be detected by the observed change that they induce in the electron density on the surface, which results in a shift in the surface plasmon absorption maximum. This is the basis for the use of noble metal nanoparticles as sensitive sensors.

2.2.1.1 Application of LSPR absorption of NPs for chemical analysis [14]

Recently, noble metal nanoparticles-based UV–visible spectrometric methods have drawn special attention for their selective and sensitive reorganization of target species (inorganic, organic and biomolecules) in various complex matrices. Metallic NPs (Au and Ag NPs) are emerging as promising analytical colorimetric reporters for a wide variety of analytes because of their intrinsically exploitable properties such as the high extinction coefficient and the distinct variation in color based on their dispersion and aggregation states. As a result, noble metallic NPs have been used as promising coloring probes for selective, on-site and real-time colorimetric sensing of a wide variety of molecules in various matrices that facilitates visualization of targets species directly with naked eye. In recent years, AgNPs-based signal amplifications hold great promise in the development of sensitive and selective miniaturized UV–visible approaches for real-time monitoring of trace-level target species in complex samples. For example, Ag NPs are functionalized with various organic derivatives such as p-sulfonatocalix, arene, oligonucleotide, p-nitroaniline dithiocarbamate, and 4,4-bipyridine and used as colorimetric sensors for detection of various organic molecules (pesticides, amino acids and DNA).

2.2.1.2 Application of LSPR scattering of NPs for chemical analysis [15]

Based on the macroscopic fluctuation theory and Mie theory, the intensity of scattered Rayleigh light is in proportion to λ_0^{-4}. In fact, the RLS spectrum is a particular synchronous scan spectrum at $\Delta\lambda = 0$nm (i.e. $\lambda_{excitation(Ex)} = \lambda_{emission(Em)}$). On the other hand, it is well known that there is often a peak at the double excitation wavelength ($2\lambda_{Ex}$) named the second-order scattering in the fluorometric determination which is regarded as an interference peak and usually eliminated. Actually, the SOS spectrum is an emission spectrum.

Scattering of light radiation by a solution containing dispersed particulate matter is a well-known phenomenon in optical detection methods and is used in nephelometric detectors to measure the scattered radiation at a 90° angle to that of the radiation source. A similar phenomenon occurs in spectrofluorimetric detectors due to diffraction of light from the monochromator grating which passes through the emission monochromator and is detected as a peak at twice the excitation wavelength. This diffraction is called second-order diffraction of scattered excitation light (or second-order scattering–SOS), and it is a non-linear optical scattering

phenomenon that has been typically dealt as an unwanted source of light and suppressed or eliminated by using optical long-pass filters. The intensity of second-order scattered light, in analogy to nephelometry, is proportional to the concentration of the scattering particles provided that they are sufficiently separated from each other. On the basis of this principle, several studies have used SOS as an analytical detection strategy after the target analytes reacted with specific reagents in order to form a suspension of ion association aggregates which are large enough to scatter the incident light. This approach has been applied to the determination of micro-amounts of therapeutic drugs, bio-macromolecules such as nucleic acids and proteins, anti-coagulation agents, food additives and as an alternative detection strategy for non-fluorescence compounds (e.g. oligomers, saccharides, etc) in liquid chromatography. Recently, scattering of light at double the incident wavelength has been observed in concentrated solutions of organic and inorganic nanoparticles and has been utilized for the investigation of nanoparticle properties in standard solutions or as a template for the detection of analytes that can interact with nanoparticles and induce their aggregation.

2.2.2 Preparation of nanoparticles [16]

Generally, all of the nanoparticle synthesis methods can be separated into two main types: top down and bottom up approaches. In the top down approach, the method starts with a standard bulk material for generating the nanomaterial, whereas in the bottom up approach, the method starts from atoms or molecules and builds them up into NPs, as shown in Figure 2.5. In another classification, methods of nanoparticle synthesis are grouped into two main types: physical method and chemical reduction method.

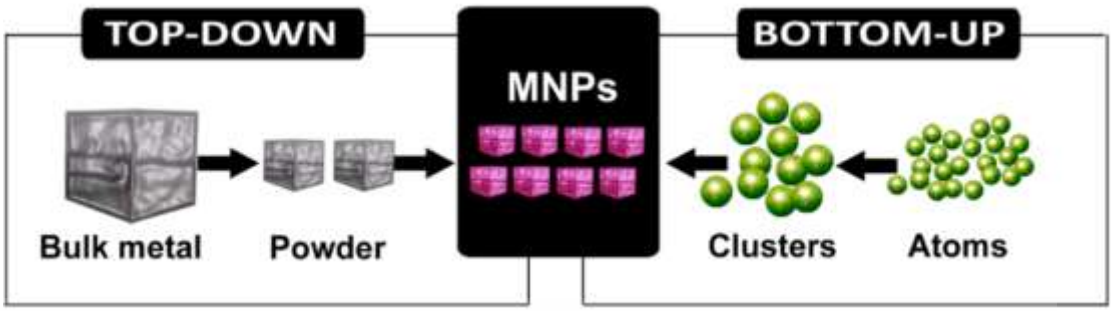


Figure 2.5 Schematic representation of the top-down and bottom-up approaches for the manufacturing of metal nanoparticles, adopted from [16].

Many chemical and physical methods for M-NPs preparation have been reported. In general, formation of metal nanoparticles provides favorable energy for particle growth, but if this activation energy is removed (for example, by decreasing the temperature of the system), the growth will be interrupted, thus it is possible to kinetically control the size of formed particles. One example of physical methods is evaporation of metal by heating or irradiating with laser. A disadvantage of this method is that the M-NPs will be generated without a coating, thus have a high surface energy. This leads to their immediate aggregation into polycrystalline powder form thermodynamically favored by metal-metal bonds formation.

On the other hand, the chemical method is based on the presence of stabilizer in the solution of metal salt. Faraday, in 1857, was the first to publish about a method of chemical reduction of a metal salt to form metal colloids in water. However, it was a century later that Turkevich described the first standard procedure for preparing colloidal gold reduced and stabilized by citrate. Suitable reducer choice is very important since its reductive ability determines the kinetics of M-NPs formation. The driving force in a metal reduction reaction is the redox potential difference, ΔE. The magnitude of ΔE determines the equilibrium constant value of the reaction *(Ke)* as expressed in the Nernst equation at chemical equilibrium:

$$lnK_e = \frac{nF\Delta E}{RT} \qquad 2.5$$

where *n* is the number of electrons transferred, *F* is Faraday constant, *R* is general gas constant and *T* is temperature.

Reduction is thermodymically possible only when ΔE >0, meaning that the reducing agent's redox potential is smaller than that of the metal species, and this difference in redox potentials must be higher than 0.3-0.4 V. Thus, strong reducing agents, such as borohydride, tend to form small-sized M-NPs rapidly, while weak reducers, such as alcohols, reduce the metal precursor at a slower rate producing M-NPs with larger sizes [17].

2.2.2.1 Chemical reduction method [18].

The most popular chemical approaches including chemical reduction employ a variety of organic and inorganic reducing agents. They are popular methods for synthesis of NPs because they are very simple and low cost. AgNPs can be synthesized by reducing silver ion with many types of reducing agents (e.g. borohydrides, hydrazine, formaldehyde and alcohol)

Successful chemical syntheses with many types of metal salt precursor, solvents as well as reducing and stabilizing agents have been reported.

- Metal salt precursor

Silver salt precursors are used in a bottom-up technique to produce ionic silver which can be reduced and precipitated to form NPs. Figure 2.6 shows that $AgNO_3$ is the most widely used salt precursor, accounting for almost 83% of those reported in studies of general and specific synthesis methods. The dominant use of $AgNO_3$ can be attributed to its low cost and chemical stability when compared to other types of silver salts.

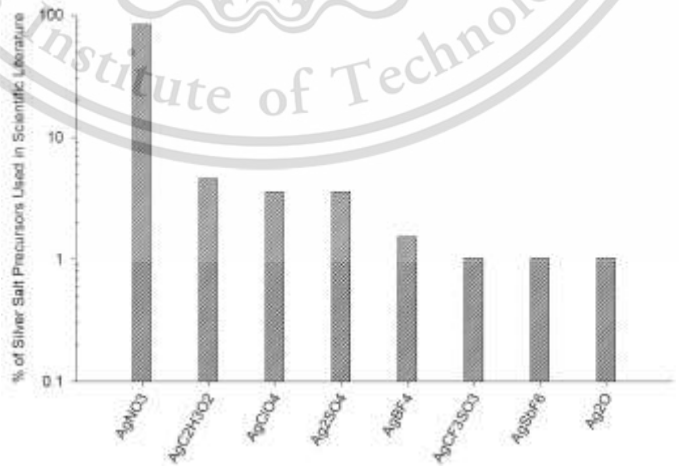


Figure 2.6 Analysis of silver salt precursors reported in studies of AgNPs synthesis [18].

- **Solvents**

Solvents are used to solubilize silver salts and other chemicals in the synthesis process. Although many organic and inorganic solvents are used in synthesizing silver nanomaterials, approximately 80% of the reported synthesis attempts use water as solvent (Figure 2.7)

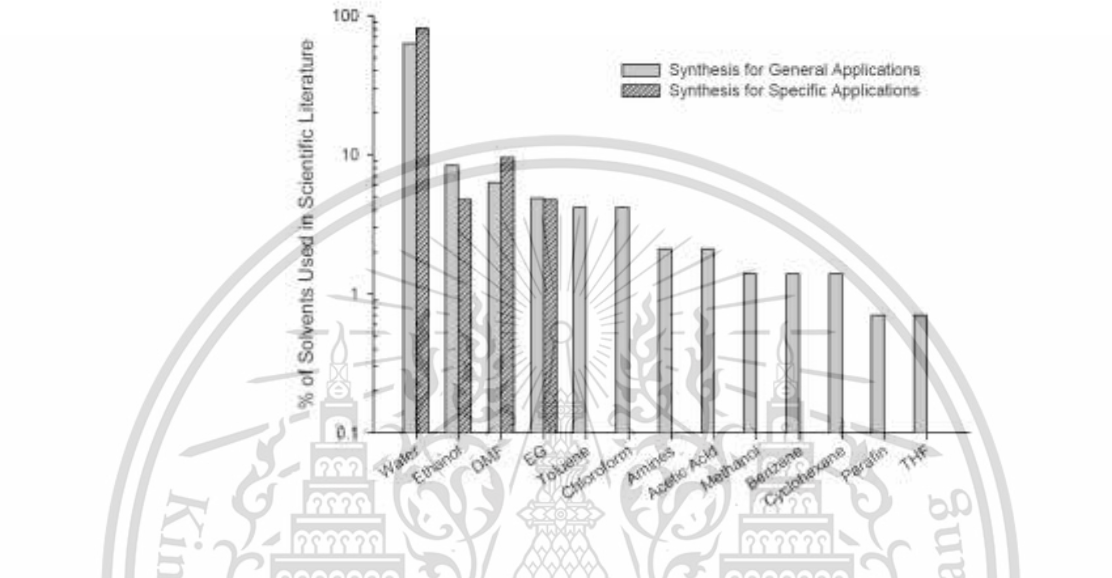


Figure 2.7 Analysis of solvents reported in studies of AgNPs synthesis [18].

- **Reducing Agent**

In the context of a bottom-up technique, a reducing agent can be a chemical agent, plant extract, biological agent, or irradiation method that provides free electrons needed to reduce silver ions and form AgNPs. A strong reducing agent such as sodium borohydride ($NaBH_4$) tends to produce a narrow range of small monodispersed particles while a weaker reducing agent such as ascorbic acid produces larger-sized particles. A critical appraisal of the scientific literature embodied in the peer-reviewed articles suggests that $NaBH_4$ (~23%) and sodium citrate (~10%) accounted for 33% of all of the reducing agents used (Figure 2.8). Smaller percentages of other reducing agents were used in special synthesis applications.

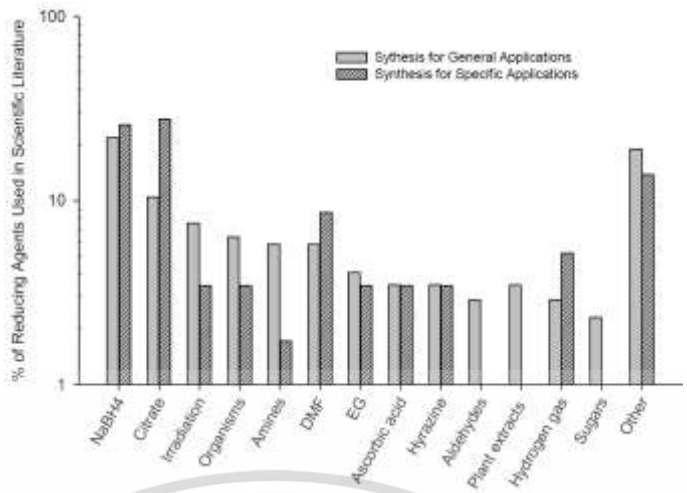


Figure 2.8 Analysis of reducing agents reported in studies of AgNPs synthesis [18].

- Stabilizing Agent

A stabilizing agent (also known as capping agent) is used in the synthesis process to prevent NPs from aggregating and thus control the size of the final product. Agglomeration is mainly caused by excess surface energy and high thermodynamic instability of the surface of NPs. A stabilizing agent relies on electrostatic repulsion force caused by surface charges or steric hindrance or both shown in Figure 2.9

Electrostatic stabilization mechanism involves the clouding of ions and counter ions around the surface of particles. The electrostatic repulsion from these ionic clouds can keep particles from aggregating.

Steric hindrance can be achieved by adsorption of hindrance molecules such as a branched polymer on the surface of M-NPs. Polymer chains on M-NPs surface produce steric repulsion which separates particles from each other [17]

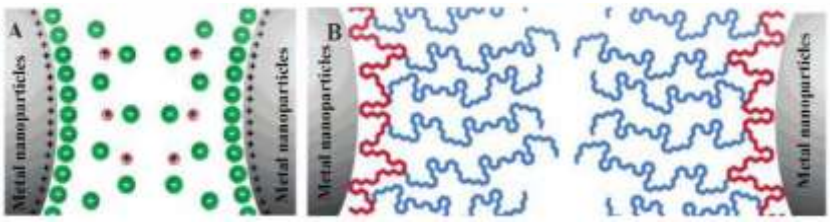


Figure 2.9 The stabilization mechanism of AgNPs; (a) electrostatic stabilization and (b) steric hindrance [17].

Consequently, there is a wide range of selection of stabilizing agents to choose from, as shown in Figure 2.10 These Include surfactants such as sodium dodecyl sulfate (SDS) or ligands and polymers that contain functional groups such as thiol (-SH), cyano (-CN), carboxyl (-COOH) and amino ($-NH_2$). The selection of stabilizing agent depends on the application. For example, polynisopropylacrylamide (PNIPAM) is commonly used as a temperature-sensitive polymer possessing a lower critical solution temperature (LCST). Below the LCST, it is hydrophilic and soluble in aqueous solution; however, the polymer becomes hydrophobic, insoluble and aggregates in solution when the temperature is raised above the LCST. Thus, AgNPs capped by PNIPAM allow for combined surface plasmon and thermal switching applications. In addition to stability, reactivity, solubility, particle shape and size are determined by the concentration of a given type of a stabilizing agent. For instance, smaller particles are obtained by increasing the carboxylate stabilizer concentration. The catalytic properties of silver nanomaterials can also depend on the type of stabilizers used. For example, cetyltrimethylammonium bromide (CTAB) or SDS decreases the adsorption of reactants to the surface of AgNPs. Commonly used synthesis methods usually produce negatively charged AgNPs; however, the use of branched polyethyleneimine (BPEI) results in positively charged AgNPs that show greater surface enhanced Raman scattering (SERS) activity over negatively charged AgNPs (reduced via citrate) for detection of thiocyanate and perchlorate ions.

As demonstrated in Figure 2.10 a review of the scientific literature revealed that sodium citrate (27%) was the most commonly used stabilizing agent, followed by polyvinyl pyrrolidone (PVP) (18%), then amines (8%). Cetyltrimethylammonium bromide (CTAB), polyvinyl alcohol (PVA), sugars and amides each accounted for 5% of the stabilizing agents used in the papers reviewed. The "other" category in Figure 2.10 represents 23% of all stabilizing agents with individual chemicals being used in only one study. It should be noted that sodium citrate was used in 50% of all of the stabilizing agents used, followed by PVP (18%), then CTAB / amines / amides / fatty acids with each representing less than 10% of used agents [18].

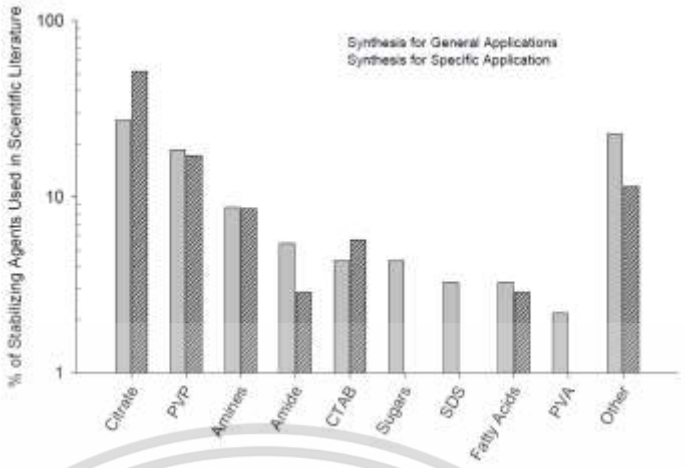


Figure 2.10 Analysis of stabilizing agents reported in studies of AgNPs synthesis [18]**.**

1) Citrate reduction of citrate and/or borohydride [19]

For example, chemical reduction of silver salts to form NPs has been achieved by using sodium citrate and/or borohydride. The use of sodium borohydride (a strong reductant) usually results in a formation of somewhat monodispersed smaller-sized AgNPs while the use of only citrate (a weaker reductant) usually results in a formation of somewhat polydispersed larger-sized AgNPs because of its slower reduction rate. The reduction reaction of silver ion by sodium citrate is shown below.

$$4Ag^{+} + C_6H_5O_7Na_3 + 2H_2O \text{ -----> } 4Ag^{0} + C_6H_5O_7H_3 + 3Na^{+} + H^{+} + O_2 \quad (2\text{-}6)$$

Reactions (2.7), (2.8) and (2.9) are individual and overall reaction steps in the formation of AgNPs upon reduction with sodium borohydride.

$$2Ag^{+} + 2e\text{-} \text{ -----> } 2Ag \quad (2.7)$$

$$2BH_4^{-} \text{ -----> } B_2H_6 + H_2 + 2e\text{-} \quad (2.8)$$

$$2AgNO_3 + 2NaBH_4 \text{ -----> } 2Ag + 2H_2 + B_2H_6 + NaNO_3 \quad (2.9)$$

Solomon et al., proposed a mechanism of nanoparticle formation based on sodium borohydride reduction and stabilization (without stabilizing agent). The nanoparticle formation is based on temporary stabilization of smaller-sized AgNPs by excess BH_4^{-} species. Figure 2.11 shows the structure of stabilized AgNPs with a shell of excess

borohydride anion. However, with time, the stabilized shell around the NPs will collapse and cause the NPs to aggregate which is largely attributed to the degradation of BH_4^- accompanied by hydrogen gas evolution as mentioned in equation 2.10

$$BH_4- + 4H_2O \text{ -----> } B(OH)_4 + 4H_2 \quad (2.10)$$

Figure 2.11 AgNPs stabilized by repulsive forces generated by borohydride anion [19].

2.2.2.2 Silver mirror reaction [20]

Justus von Liebig invented silvered glass mirror in 1835. Since then, the Ag mirror reaction has been widely used as a method for chemical deposition and mass production of Ag coatings on various kinds of substrates. In this reaction, the precursor for forming Tollen's reagent, $Ag(NH_3)_2OH$, is $AgNO_3$ which is then reduced by a sugar for example, glucose or an aldehyde to achieve elemental Ag shown in equation below.

$$RCHO + 2[Ag(NH_3)_2]^+ + 2OH^- \text{ -------> } RCOOH + 2Ag + 4NH_3 + H_2O$$

Successful reaction produces a shiny mirror. The reaction can quickly generate a shiny coating on the inner surface of a reaction container (for example, a test tube), so it can serve as an impressive demonstration of redox reactions and a detection method for aldehyde groups in laboratory classes. The Ag mirror reaction can also be performed with commercial silvering solutions under sonication to generate stable suspensions of relatively narrow-size-ranged quasi-spherical AgNPs. Nevertheless, this technique does not provide shape control, hence its use is usually limited to synthesis of high-quality Ag nanostructures. Notwithstanding, the Ag mirror reaction is still being used in a number of specific applications such as coating of big

telescopes, formulation of different kinds of Ag coating and smooth Ag surfaces with microstructure or nanostructure. For example, the reaction has been adapted for preparing Au-Ag alloyed thin films for conventional SPR sensing as well as smooth Ag films for use in micro contact printing of alkane thiols. The morphology of these films is typically granular, and as a result, different microstructures and micro patterns, such as leaf or flowerlike fractal structures, can also be created. It is also practical to use the Ag mirror reaction to coat a variety of micro and nanoscale objects with small particles or continuous thin films of Ag, including fiber optic probes, silica beads, polymer capsules, and semiconductor particles for photocatalytic applications.

2.2.2.3 Polyol process [21]

Polyol synthesis was originally developed by Fiévet and coworkers as a simple and versatile route to producing colloidal particles of metals and alloys, with typical examples including Ag, Au, Cu, Co, Ir, Ni, Pd, Pt, Ru, CoNi, and FeNi. The primary reaction of this process involves the reduction of an inorganic salt (the precursor) by polyol at an elevated temperature. PVP is commonly added as a stabilizer to prevent agglomeration of the colloidal particles. The reasons for the popularity and versatility of this synthesis include the ability for polyols to dissolve (and solvate) many precursor salts (and ions), their highly temperature-dependent reducing power, and their relatively high boiling points (for ethylene glycol, it is about 196 °C). In particular, the temperature-dependent reducing power of polyols makes them ideal for synthesis of colloidal particles (usually quasi-spherical in shape) over a broad range of sizes, as it gives one the ability to control their nucleation and growth processes through careful regulation of reaction temperature. Furthermore, the use of solvents with high boiling points allows for production of colloidal particles from some more reactive (and hence less reducible) metals such as Co, Ni, Cd, Bi, and Pb by thermally decomposing appropriate precursors. The polyol process that was used for producing silver colloid is mainly based on ethylene glycol, which serves as a good solvent for both $AgNO_3$ and PVP, because of its relatively high dielectric constant. At an elevated temperature, ethylene glycol can reduce Ag^+ ions into Ag atoms and induce nucleation and growth of AgNPs in solution phase.

2.2.2.4 Photochemical and radiation-chemical reduction [17]

Synthesis of metal NPs by photochemical (photolysis) and radiation chemical (radiolysis) is a process associated with generation of strong reducing

agents such as electrons, radicals and excited species. These two methods are only different in the amount of energy involved in the process; photochemical involves energy below 60 eV, but radiation-chemical involves energy around 103-104 eV. Using these methods to produce NPs has many advantages such as absence of impurities, high-purity NPs, and low temperature process.

Photochemical and radiation-chemical in solution is frequently employed for synthesizing metal NPs. These NPs are synthesized from solution of metal dissolved in water, alcohol and organic solvent. Under the action of light, active species are formed:

$$H_2O_{(l)} \xrightarrow{h\nu} H^+ + OH^\bullet + e^-,$$

An electron-donating solvent interacts with metal ion to produce metal particles:

$$M^+ + e- \longrightarrow M$$

Light induces not only formation of NPs but also aggregation of NPs. The aggregation can be attributed to the exchange of electric charge which gives rise to electrical force that attracts one particle to another.

2.2.2.5 Electrochemical method [17]

This method uses two electrodes and an electrolyte solution. The anode contains bulk metal which will be converted into metal NPs. An example of supporting electrolyte is tetraalkylammonium salt, which also serves as stabilizer for the metal NPs. The overall process is shown in Figure 2.12 First, the bulk metal is oxidized at the anode into metal ions. Then, the metal ions migrate to the cathode, and reduction takes place at the cathode. Consequently, formation of metal NPs takes place. The obtained NPs are stabilized by tetraalkylammonium that prevents aggregation and precipitation of NPs. This method has an advantage of producing NPs of high purity, and specific particle size can be controlled by adjusting current density.

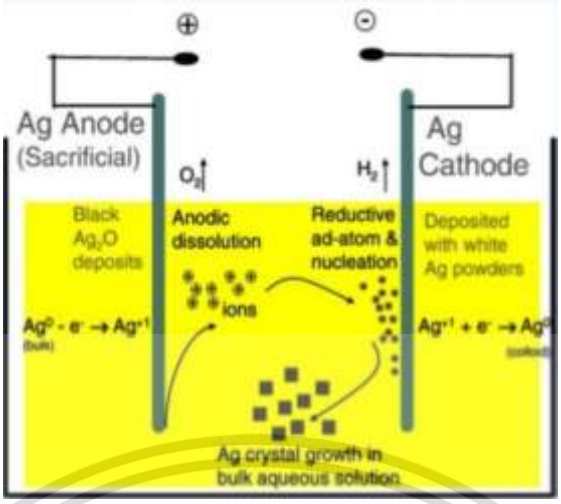


Figure 2. 12 Schematic of the Apparatus for synthesis of AgNPs by electrochemical method [22].

2.3 Sequential Injection Analysis (SIA) [23]

Sequential injection analysis (SIA) was established in 1990 by Ruzicka and Marshall at the University of Washington. This methodology was proposed as a new feasible and mechanically simpler alternative to flow injection analysis (FIA) for handling solutions. This technique for automatic sample analysis is based on the same principles as FIA (controlled partial dispersion and reproducible sample handling), and it offers different possibilities with a series of advantages and disadvantages in relation with its parent technique. The advantage of SIA over FIA is that SIA typically consumes less than one-tenth the reagent and produces far less waste, an important feature when dealing with expensive chemicals, hazardous reagents, or online/remote site applications. Disadvantages of SIA are that it tends to run slower than FIA and has some major difficulties in the mixture of sample and reagents. SIA is a single-line system completely controlled by microcomputer that can be configured to perform most operations that a conventional FIA can, with no or minimal physical reconfiguration of the manifold, allowing it to perform determinations of different analytes easily. SIA has proven to be a technique that can be designed to operate in a multi-parametric way.

2.3.1 Principles of operation and SIA components

The sample volume in a conventional FIA manifold is inserted into a carrier stream and subsequently merged downstream with reagents. However, SIA is a fully automated discontinuous flow technique based on the sequential aspiration of precise volumes of sample and reagent(s) in a holding coil, which are afterwards dispersed into the reaction coil by flow reversal. As a consequence of the sequential and discontinuous operation, the injection frequency as well as the consumption of reagents and sample is evidently reduced compared to FIA. The most basic SIA system comprises an automatic bi-directional pump or syringe, a multi-position selection valve, a reactor and holding coil, a detection system, and finally a computer that controls the functions of each component. A schematic diagram showing the main parts of the instrumentation and the principle of measurement is shown in Figure 2.13

The SIA system is initially filled with a carrier stream into which a zone of sample and a zone of reagent(s) are sequentially aspirated into a holding coil. In this way, a stack of well-defined zones is obtained. By means of a flow reversal, a composite zone is formed in the holding coil, as sample and reagent zones penetrate mutually, due to the parabolic profile induced by differences between flow velocities of adjacent streamlines and combined axial and radial dispersion. flow reversal and flow acceleration further promote mixing. The multi position valve is then switched to the detector position, and the flow direction is reversed, propelling the sample/reagent combined zones through the detection system, where the reaction product is monitored.

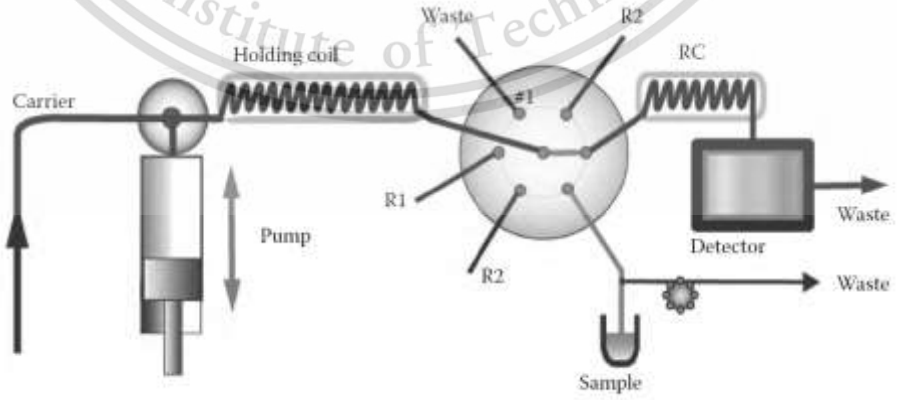


Figure 2.13 Schematic manifold of sequential injection analysis (SIA) system [23].

2.3.1.1 Pump

According to Ruzicka and Marshall, the pump together with the valve serves as a precision volumetric transport device and should have zero inertia and zero elasticity, requirements that preclude the use of peristaltic pumps shown in Figure 2.14 Thus, any computer-controllable piston pump capable of forward and reversed movement, would be suitable. The requirements for the pump are that it is precise, reproducible, bi-directional, and able to measure small volumes. Computer control is imperative. The low-pressure syringe pump for liquid driver were introduced by Gübeli et al. in 1991. These pumps provided a sinusoidal flow as a result of non-uniform motion of the piston in the cam-driven piston pump, giving rise to a flow that is not constant but reproducible. Some researchers have used a peristaltic pump, its main advantage being higher analytical frequency as there is no need to aspirate wash solution, in contrast to syringe pumps which require priming before use and have a limited reservoir capacity. The disadvantage of the peristaltic pump arises from the need of fairly elastic tubes which have a much shorter life. In order to circumvent this inconvenient, Cladera et al. proposed an auto-burette to propel the flow in SIA. Nevertheless, robust syringe pumps as liquid drivers rather than other flow pumps have been the most widely used to aspirate zones and propel the stack of zones through the detector because they enable the manipulation of sample and reagent volumes at low μL level with high precision.

Figure 2.14 Syringe pumps [24].

2.3.1.2 Selection Valve

In SIA, the multi-position selection valve shown in Figure 2.15 which facilitates the use of different chemistries without reconfiguration of the

manifold, is the heart of the system. Small dead volume and zero cross contamination between ports are essential features of a good selection valve. The multi-position valve can be connected to various sample and reagent containers, typically via Teflon tubing. Its central port (central communication line or communication channel) is connected to a high precision stepper motor syringe pump that is used to aspirate well-defined volumes of sample and reagent solutions into the holding coil from their containers. Under precise computer control, the selection valve alternately chooses sample and reagent and stacks them into a tubular conduit. The electrically activated selection valve must allow random access of the ports. Usually, valves are available with between 6 and 28 ports, but a 10-port multi-position valve is by far the most widely used in SIA systems.

Figure 2.15 (a) Internal of 8- port-multi-position selection valve; (b) External of 8- port-multi-position selection valve [24].

2.3.1.3 Reactor and Holding Coil

The reaction coil is the part connecting the selection valve with the detector shown in. This component is varied depending on the involved chemistry and the amount of dispersion required. However, the reaction coil is usually kept as short as possible to avoid excessive dilution of the formed product zone. As used in FIA systems, the most suitable material for making reactor coils is polytetrafluoroethylene (PTFE) because this material is chemically resistant and adsorbs the least solutes on its surface. The holding coil is placed between the pump and the common port of the multi-position selection valve. This additional coil is made from PTFE tubing wound around a plastic tube. The main function of this tubing

is as a holding reservoir into which sample and/or reagent is sequentially aspirated. The holding coil volume should be large enough to prevent the stack of zones from being forwarded from the holding coil to the detector before the chemical reaction is taken place. It is essential to investigate the optimum tubing size and length to assess the best sensitivity and precision of the SIA system.

2.3.1.4 Detection System

UV-Vis spectrophotometry is the most frequently used detection technique in SIA systems, but in the last few years it has been closely followed by other techniques such as, fluorescence spectrophotometry, atomic absorption (AA), inductively coupled plasma (ICP), inductively coupled plasma-mass spectroscopy (ICP-MS), and electrochemical techniques such as adsorptive cathodic stripping voltammetry (ACSV) or anodic stripping voltammetry (ASV) and potentiometry. As for FIA methodologies, the only requirement to couple a detection system to a SIA manifold is that in the case of non destructive detectors, they be equipped with a flow cell. In addition, low dead-volume and immunity to bubbles are key requirements.

2.3.1.5 Computer

One of the main drawback of SIA methodologies is the necessity of controlling the overall analytical instrumentation and physical variables (namely, volumes and flow-rates) by available computer programs. Thus, the core of an SIA manifold is the flow program. Microprocessor control with appropriate software is imperative to control the flow direction, rate and timing of the pump, the position of the multi-port valve and to collect, control and process the data. In this sense, many SIA procedures were proposed with in-house built software developed in LabVIEW, Microsoft QuickBasic, MATLAB, Microsoft Visual Basic, etc., though several commercialized DOS or Windows based software packages are also available: FIALab (FIAlab Instruments, USA), the FlowTEK package designed by Marshall and coworkers (MINTEK, Randburg, South Africa), Atlantis (Lakeshore Technology, Chicago, IL, USA), the DARRAY package developed by the research group led by Prof. Cerdà at the University of the Balearic Islands, Spain), etc.

2.4 Literature Review

2.4.1 Methods for determination GABA

2.4.1.1 HPLC

Amir Hayat et al. [2] examined the GABA and lysine contents in Pakistani rice varieties. The two analytes were detected by using Schiff base derivatives with 2-hydroxynaphthaldehyde. The separation on HPLC system was accomplished using a reverse phase C-18 column with Diode Array Detector at 254 nm. The spectrophotometric study has shown that the best reaction conditions were at pH 8 and 80 °C with a derivatizing time of 10 min. The amount of 2-hydroxynaphthaldehyde reagent was selected to be 1.5% (w/v) which was enough for complete consumption of the standard during the reaction with highly stable. The calibration curves were found to be linear over a concentration range of 3.83-34.58 mg mL^{-1} for GABA and 5.16-48.68 mg mL^{-1} for lysine with a correlation coefficient of 0.998 for both standards. GABA and lysine contents were found to be higher in brown rice varieties (4.1-6.58 mg/100 g for GABA and 15.1-27.6 mg/100 g for lysine) than the rice varieties that had been milled (0.32-0.47 mg/100 g GABA and 13.1-19.8 mg/100 g lysine). The reaction of the standard with the derivatizing reagent was quantitative and reproducible. The derivative seemed to be quite stable, provided high sensitivity in the UV region, and separated easily with a simple gradient elution system of methanol and water. The selectivity and reproducibility of the method allowed determination and separation of GABA and lysine from the plant seeds in less than 10 min.

M.Y. Khuhawar and A.D. Rajper [25] investigated the use of 2 hydroxynaphthal-dehyde for selective HPLC determination of GABA from human cerebral spinal fluid. GABA was determined by, firstly, precolumn derivatization with 2-hydroxynaphthaldehyde then elution was made using Phenomenex C 5 mm column with methanol:water (62:38 v/v) and UV detection at 330 nm. Glycine, l-lysine, tyramine and GABA mixture in samples were able to be separated completely. The optimal conditions for this method were the following: derivatizing reagent 2-hydroxy-naphthaldehyde (0.3% w/v in methanol) buffer solution at pH 8 and heating time of 10 min at 80 °C. The sample solution (5 ml) was injected into HPLC. The linear calibration curve was obtained for GABA in the range of 1.12-28 mg mL^{-1} corresponding to the detection limit of 2.8 ng/injection (5 µL) and a regression coefficient of

0.998. The method was used for determination of GABA in cerebral spinal fluid.

Dihua Shangguan et al. [26] performed a fluorescence derivatization of amino acids and peptides. Amino acids (serine, glutamic acid, valine, phenylalanine, lysine and GABA) and two neuropeptides were adsorbed on the surface of a solid-phase sorbent and derivatized in organic solvents. The derivative products were eluted into a 0.5 mL centrifuge tube with 100 or 200 μL of eluant, and then 10 μL of the collected eluate was injected into the column of the HPLC system with fluorescence detection. Compared with the traditional derivatization in the liquid phase, the extent of formation of byproducts of 9-fluorenylmethylchloroformate (FMOC-Cl) with water was greatly decreased, and the excess FMOC-Cl was eliminated completely. The results show that the adsorbent did not significantly affect the reaction rate and yield of amino acids. The method is suitable for derivatization of amino acids and some peptides with FMOC-Cl and greatly reduces the interference by excess derivatization reagent and its hydrolysis byproducts.

2.4.1.2 Spectrophotometry

Guijin Zhang and Alan W. Bown [27] reported that GABA-transaminase involved in nitrogen metabolism during a plant process via GABA shunt and succinic semialdehyde dehydrogenase allowed for a spectrophotometric assay of GABA:

(1) GABA + α-ketoglutarate ------> glutamate + succnic semialdehyde;

(2) Succinic semialdehyde + $NADP^+$ ------> succinate + NADPH.

GABA values are related to the increase in absorbance due to NADPH production. The calibration graph indicates a linear relationship between the absorbance increase and GABA values that varied from 10 to 100 nmol per cuvette. The method described is rapid and inexpensive because it does not require chromatographic separation and derivatization of amino acids. It avoids overestimating GABA by eliminating synthesis subsequent to tissue sampling and eliminates pigments that interfere with the spectrophotometric assay.

2.4.1.3 Electrochemistry

Osamu Niw et al. [28] developed an on-line GABA sensor by using a glassy carbon electrode modified with a bioanalytical system: gabase-glutamate oxidase (GluOx)/ Osmium-poly-(vinylpyrridine) wired horseradish peroxidase (Os-gel-

HRP) bilayer films combined with a prereactor in which GluOx and catalase are immobilized in series. The reaction scheme of this method is as follows.

(1) GABA + **α**-ketoglutarate $\xrightarrow{\text{GABA}}$ succinic semialdehyde (SSA) + glutamic acid,

(2) glutamic acid + $H_2O \xrightarrow[\text{GluOx}]{\text{SSDH}}$ **α**-ketoglutarate + NH_3^+ + H_2O_2.

In this system, L-glutamate contained in a biological sample can also be reacted on the electrode; therefore, a small volume protractor was used in which GluOx and catalase were immobilized to consume all of the L-glutamate molecules in the sample solution. Since the oxidation of L-glutamate produces **α**-ketoglutarate, GABA could be measured without adding any reagents containing **α**-ketoglutarate in the sample when the L-glutamate concentration was relatively high. The sensor exhibited a sensitivity of 1.56 nA μM^{-1} for and a detection limit of 0.1 μM with a linear range of 0.1-10 μM for GABA which can be measured in the absence of **α**-ketoglutarate when there is L-glutamate in the sample solution. The sensor showed a high selectivity against most other neurotransmitters including glutamate, which also reacts on the modified electrode.

2.4.2 Nanoparticles-based sensor

Jian Linga et al. [29] developed a determination of berberine hydrochloride with citrate-capped AgNPs by using colorimetric method. Typically, a colloidal solution of AgNPs is yellow owing to its surface plasmon resonance absorption peak at 396 nm. Citrate capped AgNPs with negative charged surface can be dispersed in water symmetrically by the electrostatic repulsion between each particle. However, the positive berberine would induce the aggregation of AgNPs via electrostatic attraction. It was found that the color of the colloid of AgNPs would change from yellow to olive green and even to blue with increasing berberine concentration and depending on the degree of aggregation of AgNPs. Furthermore, there is a good linear relationship between the absorbance change of AgNPs and the concentration of berberine in the range of 0.05 to 0.4 μmol L^{-1} with a limit of determination of 1.3×10^{-8} mol L^{-1}. This colorimetric analytical method without use of any expensive equipment is so convenient, economy and speedy that it has a flourishing prospect in analytical chemistry.

Mengmeng Gao et al. [14] developed a colorimetric sensor for determination of entecavir with citrate capped AgNPs which is yellow in color owing to its surface plasmon resonance at 398 nm. Citrate-capped AgNPs with negative charged surface can be dispersed in the water symmetrically by the electrostatic repulsion between each particle. Neutralization of the electrostatic repulsion of AgNPs resulted in aggregation of AgNPs and a consequent color change from yellow to wine-red. This change provides a platform for colorimetric detection of entecavir and can be obviously observed by naked eyes. The absorption ratio was linear with the concentration of entecavir in the ranges of 5.04–25.2 μg $mL^{-1}$and 1.01– 5.04 μg $mL^{-1}$with linear coefficients of 0.9907 and 0.9955, respectively. The recoveries of 3 μg mL^{-1}, 10 μg mL^{-1}, and 20 μg mL^{-1} of entecavir were 105%, 97% and 106%, respectively. The AgNPs detection method reported in this study is very suitable for on-site screening of entercavir in human urine samples.

Sweta K. Laliwala et al. [30] designed a citrate-capped AgNPs method for colorimetric determination of rizatriptan, naratriptan, sumatriptan and zolmitriptan triptan-family drugs. The yellow-colored citrate-capped Ag NPs was monitored of its surface plasmon resonance absorption peaks at 396 nm by UV–vis spectrophotometry. After a few minutes of reaction, the color of AgNPs was changed from yellow to orange in the case of rizatriptan or to brown/red in the cases of naratriptan, sumatriptan and zolmitriptan. The new SPR peaks were at 560, 548, 520 and 570 nm, respectively. Under optimized conditions, good linear relationships (R^2= 0.9865, 0.9810, 0.9767, 0.9914) were obtained in the concentration range of 0.001–1.0 mM with limits of detections of 7.3, 8.6, 10.5 and 84.0 nM for rizatriptan, naratriptan, sumatriptan and zolmitriptan, respectively. The present method is simple, rapid and sensitive to four triptan-family drugs and can act as a multi-drug sensor. Additionally, the method was successfully validated to quantify triptan drugs in pharmaceutical samples.

Fen Ding et al. [31] investigated AgNPs on second-order scattering (SOS) and fluorescence of complexes of Tb (III) with quinolones as well as developed a determination method for quinolones (Qs). The characteristic fluorescence of Tb (III) ion was enhanced and the maximum fluorescence peak locates at 545 nm. The SOS peak at 545 nm also appears for the Tb (III)–Qs complexes with an excitation wavelength of 274 nm. When the AgNPs were added to the Tb (III)–Qs system, the luminescence intensity at 545 nm greatly increased. Pipemidic acid (PPA) enhanced

the fluorescence intensity of Tb (III) significantly, while its own fluorescence intensity at 480 nm decreased greatly, indicating that Tb (III) formed a complex with PPA, in which the ligand absorbed the ultraviolet light and was turned into excited state. The SOS peak also appeared at 640 nm in the spectra and the intensity was increased when AgNPs were added to the Tb (III) complex with PPA. Under optimized conditions, the method was linear in the range of 2.0×10^{-10} to 1.0×10^{-5} mol L^{-1} with limit of detection for PPA of 4.7×10^{-11} mol L^{-1}. The experimental results showed that a certain size and concentration of AgNPs greatly enhanced the fluorescence SOS intensity. The method was successfully applied to the determination of the content of Qs in tablet, capsule, urine and serum samples. The apparatus was cheap, and the interference was low. This was the first report that AgNPs have been added to a solution phase to determine SOS intensity.

Chien C. Wang et al. [32] synthesized AgNPs coated with EDTA (EDTA-AgNPs) by citrate reduction method and characterized them by UV–vis spectroscopy and fluorescent spectroscopy. The surface of AgNPs remained negatively charged and, in presence of counter ions, acquired electrostatic double layers. These double layers provided a repulsive force enabling the silver colloid to be stable in aqueous solution. This phenomenon is reversible once the hydroxide was removed by subsequent washing steps, obtaining a transparent solution of monodispersed NPs with an average size of 40 nm. The absorption spectrum of EDTA-AgNPs obtained had a maximum at 415 nm. The derivatized NPs showed fluorescent emission and second order scattering (SOS) signals which in presence of nitrate are both attenuated. These nanosized colloids presented an excitation and emission maximum at 225 and 295 nm, respectively. Additionally, the SOS signals presented better sensitivity than the fluorescent emission; thus, the SOS signals were chosen for nitrate determination. Under established optimal conditions, a linear response has been obtained within the range of 6.4×10^{-4} to 3.0 μg mL^{-1} nitrate concentration with a detection limit of 1.8×10^{-4} μg mL^{-1}. The obtained results showed that the EDTA-AgNPs can be applied as sensor for nitrate determination in pharmaceutical samples. The main advantage of the proposed method is the possibility of direct nitrate determination with very good accuracy, sensitivity and tolerance, without the need of prior reduction to nitrite nor any pre-treatment of the samples.

2.4.3 Application of SIA for chemical analysis

Natcha Kaewwonglom and Jaroon Jakmunee [33] developed a compact SIA and colorimetric system with capability to determine multi- parameter for determination of iron, manganese, phosphate and ammonium in water samples. The system was optimized for suitable conditions by changing parameters in the software program without reconfiguration of the hardware. The resulting iron and manganese standard solutions in the concentration range 0.2-10 mg L^{-1} were aspirated into the system for construction of calibration graph. The system was optimized for phosphate and ammonium in the range of 0.3-5 mg L^{-1}. This method achieved correlation coefficients of 0.9998, 0.9973, 0.9987 and 0.9983 and detection limits of 0.01, 0.14, 0.004 and 0.02 mg L^{-1}, respectively, for iron, manganese, phosphate and ammonium. Recoveries of the analysis were obtained in the range of 82-119%. The proposed system has good precision, uses low chemical consumption as well as provides high throughput. It is also inexpensive and portable.

S. Teerasong, et al. [34] developed a completely reagent-free module based on SIA for process analysis of carbonated soft drinks. This SIA was utilized to achieve automatic liquid handling of the samples. The SIA module was assembled with a manifold for degassing the sample. A modified membrane-less vaporization (MBL-VP) unit was adopted for degassing carbonated soft drink via stirring with magnetic bar to accelerate vaporization of CO_2. Zones of the degassed sample were alternately fed into a near infrared and red-green-blue (RGB) photometer for detection of sucrose and color contents, respectively. Detection of the light reflected at the liquid interface (schlieren effect) of sucrose and water was utilized for sucrose content measurement. A near infrared LED was chosen as the light source to ensure that all the ingredients and dyes in soft drinks would not interfere by contributing to the light absorption. At the optimum condition, a linear calibration graph of Brix value from 3.10 to 46.50 Brix (r^2 = 0.998) was obtained. The system provided reasonable recoveries ranging from 95.8 to 105% with good precision (%RSD = 3.2). The limit of detection (LOD) and limit of quantitation (LOQ) of 2.79 and 2.88 Brix were obtained for sugar, respectively. Precision diminished at sucrose concentration greater than 46.50 Brix, because of the effect of increased viscosity. The same module can be used to monitor the color of the soft drink as well as the dissolved CO_2 during production. For measuring the color, the sample is segmented between air plugs to avoid dispersion. An RGB-LED was chosen as

the light source in order to make this module applicable to a wide range of colored samples. The module also has a section where dissolved CO_2 is measured via vaporization of the gas from the liquid phase. Dissolved CO_2, in a flowing acceptor stream of water resulting in the change of the acceptor conductivity, is detected using an in-house capacitively-coupled contactless conductivity detector (C^4D). The module includes a vaporization unit that is also used to degas the carbonated drink, prior to the measurements of sucrose and color within the same system. The method requires no chemicals and is therefore completely friendly to the environment.

Marcelo S. Pinto Silva et al. [35] developed a coupling of sequential injection (SI) with monosegmented flow analysis (SIA–MSFA) for determination of Fe(II). SIA–MSFA , in which there is no dispersion of the reaction zone with carrier, that could perform in-line dilution. The proposed method was evaluated by performing dilutions with bromothymol blue (BTB) dye and applied to automate the spectrophotometric methodology for determination of Fe(II) in an anti-anemic medicine exploiting the reaction with *o*-1,10-phenanthroline. The methodology allowed mechanization of procedures for performing standard additions and constructing analytical curves by using a single stock standard solution, with very simple and conventional computation of the sample concentration. The method achieved a sampling frequency of 30 analyses per hour and a detection limit of 25 μg L^{-1}. Another advantage of coupling SIA with MSFA is the efficient mixing of sample with reagents, assuring appropriate stoichiometric excess of reagents along the reaction zone which is a difficult task to perform with conventional SIA when several reagents are involved in the method.

R.N. Fernandes et al. [36] developed a sequential injection set-up with an additional binary sampling approach. The system versatility was widened both by accomplishment of diverse analytical procedures and by facile handling of stock solutions, usually performed in a batch mode. In this way, in-line solution preparation for set-up calibration, standard addition techniques and titration procedures could be carried out without any conventional stock solution handling or modification of the physical structure of the flow system. Ion-selective electrode characterization using only one or two dilutions of the stock solutions resulted in a remarkable decrease on the reagent consumption and benchtop work for direct potentiometric measurements. Evaluation of the flow-through vitamin B_6 selective electrode and its application to

routine analysis of pharmaceuticals demonstrated the system potentialities. Determination of Vitamin B_6 in pharmaceutical products was carried out and in-line performed recoveries gave values within 97.4-103.5%. The system ability to perform titrations was ascertained using the precipitation reaction of vitamin B_6 with tetraphenylborate. Features such as lower reagent consumption with a low effluent generation volume were also achieved.

D.G. Themelis et al. [37] reported that sequential-injection (SI) analysis is a valuable tool in analytical chemistry as it can automate photometric detection of phosphate in urine samples. The automated SI setup allows on-line filtration and dilution of the samples before separation through a short monolithic column. On-line dilution was performed by aspirating a zone of sample with volume V_S from the sample line into the holding coil (HC), transferring volume (V_T) of that zone into the additional dilution coil (DC) and, next aspirating a portion of V_T with volume V_A (the volume transferred back into the HC from the DC). This dilution introduced an additional dispersion of the initial sample zone represented by an effective dispersion coefficient (D_{eff}) that can be tailored to the desired value by appropriate selection of the volumes V_S, V_T and V_A. Actual detection of phosphates was performed by the molybdenum blue method with detection at 690 nm. Under optimal conditions, a linear calibration range of 0.05×10^{-2} to 3×10^{-2} mol L^{-1} that covered all possible actual phosphate concentrations in urine samples was obtained. The regression coefficient was r^2 =0:996 and the relative standard deviation was s_r = 2.8% at 1×10^{-4} mol L^{-1} phosphate level (n=10) when applied to urine samples; the recoveries were >97%. The advantages of the proposed method are the simple manifold and instrumentation used, the wide scope for automated on-line dilution, the low consumption of sample and reagents as well as selective determination of phosphate in this matrix without any sample pretreatment.

2.4.4 This work

This work is a development of two methods for determination of GABA in food using silver nanoparticle sensor. It is divided into two parts: the first part is a colorimetric AgNPs method and the second part is a light scattering method with SI system.

2.4.4.1 The colorimetric AgNPs method

The colorimetric method was developed for determination of GABA in GABA food supplement samples. The AgNPs sensor was negatively-charged citrate-stabilized AgNPs. At an acidic pH of 3.8, GABA had positive charges due to protonation of amine groups. Therefore, GABA could induce aggregation of AgNPs by electrostatic interaction. Aggregation resulted in a color change of the suspension from yellow to green, which could be spectrophotometrically monitored. Using this method, parameters affecting the interaction of GABA and AgNPs such as surrounding medium, pH value, ionic strength, concentration of AgNPs, and reaction time were optimized and its analytical performance was determined. Using the optimized parameters were obtained, the method was applied to detection of GABA in food supplement samples. Finally, the results obtained by the developed method were validated against the results from an established spectrophotometric method with a napthaldehyde reagent.

2.4.4.2 Light scattering method with automated sequential injection

An automated sequential injection (SI) with second order light scattering (SOS) detection for determination of gamma-aminobutyric acid (GABA) was developed. The AgNPs sensor used in this method is the same one used in the colorimetric AgNPs method mentioned above. GABA induces the AgNPs to aggregate into bigger particles. This aggregation results in a change of scattered light which is monitored with a spectrofluorometer. In this part, working standard solutions of GABA were prepared in-line by an SI system pumping appropriate volumes of GABA stock solution and acetate buffer into a holding coil. Solution of AgNPs was subsequently drawn into the coil. The reaction zone was then transferred to a spectrofluorometer set with excitation and detection wavelengths at 300 and 600 nm, respectively. Flow parameters affecting the sensitivity of detection such as sequence zone, flow rate, mixing coil length, acetate buffer concentration, AgNPs concentration and AgNPs volume were optimized, and the analytical performance of the method was determined. Using the optimized parameters, the method was applied to detection of GABA in GABA food supplement and tea samples. For the determination of GABA in food supplement samples, an external calibration method was used whereas for green tea sample that contained several compounds that could interfere with the analysis, a standard addition method was used to minimize the interference. Finally,

the results obtained by the developed method were validated against the results obtained from an established HPLC method.

Chapter 3

Research methodology

3.1 Chemicals

All chemicals were of analytical grade. Deionized (DI) water (Zeneer Up 900, Korea) was used in all experiments. All Chemicals used in this research are shown in Table 3.1.

Table 3.1 List of chemicals and their suppliers.

Chemicals	Formula	Assay (%)	Suppliers
Gamma-aminobutyric acid (GABA)	$C_4H_9NO_2$	≥99.0	Sigma-Aldrich, China
Silver nitrate	$AgNO_3$	99.88	Carlo Erba, UK
Tri-sodium citrate dihydrate	$C_6H_5Na_3O_7.2H_2O$	99.0	Sigma-Aldrich, Japan
Sodium borohydride	$NaBH_4$	99.0	Sigma-Aldrich, Germany
Sodium acetate trihydrate	$CH_3COONa.3H_2O$	99.5	Rankem, India
Acetic acid	CH_3COOH	99.5 - 105.0	Carlo Erba, UK
Sodium hydroxide	NaOH	98.0	Rankem, India
2-hydroxynaphthaldehyde	$C_{11}H_8O_2$	≥95% (HPLC)	Aldrich, USA
Boric acid	H_3BO_3	99.0	Sigma, USA
Potassium chloride	KCl	99.5	Merck, USA
Methanol	CH_3OH	98.0	Carlo Erba, UK

3.2 Materials and apparatus

3.2.1 Materials

In this work, two samples of GABA food supplement and two samples of instant green tea were analyzed. The supplements were purchased from local drug stores. The green tea samples were from Japan, purchased via the Internet.

3.2.2 UV-visible spectrophotometer (UV-vis)

A UV-visible spectrophotometer (UV-vis) model V-630 (Jasco, USA.) was used to characterize silver nanoparticles (AgNPs) and to measure the surface plasmon resonance (SPR) band of AgNPs.

3.2.3 Transmission electron microscope

The morphology and size of AgNPs were determined by transmission electron microscopy (TEM). TEM Images were taken with a Tecnai G^2 20 electron microscope (FEI, USA.) operating at 200 kV.

3.2.4 Zeta potential analyzer

The zeta potentials of AgNPs were determined by using a zeta potential analyzer (Malvern Instruments, UK).

3.2.5 Devices in the sequential injection system

The sequential injection system comprises a syringe pump (SP) (Hamilton Company, Reno, NV) equipped with a 5-mL syringe and an eight-port selection valve (SV) (Hamilton Company), a holding coil (HC) made of polytetrafluoroethylene (PTFE) tubing (1.0 mm i d. x 50 cm) connected to the pump and the center port of the SV, a mixing coil of another 50 cm of the same size of tubing (MC) and A Jasco FP-8200 spectrofluorometer (Jasco Corporation, Tokyo, Japan) with a Hellma 176.052QS flow-through cell (Hellma Analytics, Müllheim, Germany).

3.3 Preparation of chemicals

3.3.1 Silver nitrate solution

$AgNO_3$ (0.64 mM) was freshly prepared by dissolving 0.0109 g of $AgNO_3$ in 100 mL of DI water.

3.3.2 Sodium citrate trihydrate solution

Sodium citrate trihydrate (5 mM) was prepared by dissolving 0.1471 g of sodium citrate trihydrate in 100 mL of DI water.

3.3.3 Sodium borohydride solution

$NaBH_4$ (0.1% w/v) was freshly prepared by dissolving 0.025 g of $NaBH_4$ in 25 mL of DI water.

3.3.4 Acetate buffer solution

The preparation procedure of 0.5 M acetate buffer (pH 3.8) was as follows: (a) prepare 0.5 M solution of sodium acetate trihydrate (34.02 g in 500 mL DI water) and (b) prepare 0.5 M solution of glacial acetic acid (14.29 mL in 500 mL DI water); then, add 50 mL of solution (a) to 450 mL of solution (b) and adjust the pH of the mixture to 3.80 with 1 M NaOH.

3.3.5 2-Hydroxynaphthaldehyde solution

2-Hydroxynaphthaldehyde (0.3% w/v) was prepared by dissolving 0.15 g of 2-hydroxynaphthaldehyde in 50 mL of methanol and kept in the dark.

3.3.6 Borax buffer solution

The preparation procedure of borax buffer (pH 8) was as follows: (a) prepare 0.1 M solution of boric acid by dissolving 0.3100 g of it in 50 DI water and (b) prepare 0.1 M solution potassium chloride by dissolving 0.3728 g in 50 DI water; then, mix (a) and (b) and adjust the pH of the mixture to 8 with 1 M NaOH or HCl.

3.3.7 GABA stock solution

A 2000 mg L^{-1} GABA stock solution was prepared by dissolving 0.2 g of GABA in 100.0 mL of DI water. A series of working standard solutions was prepared by pipetting various aliquots of the stock solution into 25-mL volumetric flasks, adding 3.0 mL of acetate buffer and then adding appropriate DI water volumes to the mark. All volumes and final concentrations of standard GABA solutions which were used in Part 1 (colorimetric AgNPs method) are shown in Table 3.2.

Table 3.2 Preparation of GABA standard solutions for the colorimetric AgNPs method (Part 1).

Volume of 2000 mg L^{-1} GABA solution (mL)	Volume of 0.5 M acetate buffer (pH 3.8) (mL)	Final volume in 25-mL volumetric flask (mL)	Final concentration of standard GABA solution (mg L^{-1})
1.250	3	25	100
1.875	3	25	150
2.500	3	25	200
3.750	3	25	300
5.000	3	25	400
6.250	3	25	500

Note: Every GABA standard solution was incubated overnight prior to use.

3.4 Preparation of AgNPs

AgNPs were prepared by reducing $AgNO_3$ with $NaBH_4$ and stabilizing the resulting nanoparticles with sodium citrate trihydrate [38]. Briefly, 1.0 mL of 5 mM sodium citrate was added to 39.0 mL of 0.64 mM $AgNO_3$ and stirring for 20 min. Then, 10 mL of 0.1 % w/v $NaBH_4$ was added drop by drop under vigorous stirring. The reaction produced a dark colloidal suspension of which the color changed to bright yellow in an hour. The concentration of the as-prepared AgNPs was 0.5 mM based on the concentration of $AgNO_3$ solution used for their preparation. AgNPs were stored in the dark at 4.0 ± 2.0 °C in a refrigerator. Stored this way, the AgNPs were stable for several weeks.

3.5 Characterization of AgNPs

3.5.1 UV-vis spectroscopy

3.5.1.1 Five mL of 300 mg L^{-1} standard GABA solution was mixed with 5 mL of 0.2 mM of AgNPs, the mixture was vortexed for 20 seconds. Its absorbance in the wavelength range of 300 - 600 nm was determined with a UV- vis spectrophotometer in 60 seconds after mixing.

3.5.1.2 The procedural steps in 3.5.1.1 and 3.5.1.2 were repeated but with DI water as blank instead GABA standard solution.

3.5.2 Transmission electron microscopy

3.5.2.1 Five mL of 300 mg L^{-1} standard GABA solution was mixed with 5 mL of 0.2 mM of AgNPs and vortexed for 20 seconds then left standing for 60 seconds.

3.5.2.2 The mixture was dropped onto a copper grid and the excess was wiped off. The grid was then left standing for 5 minutes before it was put in a desiccator. A blank was prepared by following Step 3.5.2.1 and 3.5.2.2 but with DI water instead of GABA standard solution.

3.5.2.3 The morphology of the prepared copper grid was observed by transmission electron microscopy.

3.5.3 Zeta potential analysis

Five mL of 300 mg L^{-1} standard GABA solution was mixed with 5 mL of 0.2 mM of AgNPs having been stabilized by 1 mL of 5 mM citrate when it was prepared. The mixture was vortexed for 20 seconds. Its zeta potential was determined in 60 seconds after that by a zeta analyzer in the potential range of ± 100 mV.

Part 1 Colorimetric AgNPs method

The colorimetric method was used with an external calibration for determination of GABA in food supplement samples.

3.6 Optimization of colorimetric AgNPs method for GABA analysis

3.6.1 The effect of stabilizer (sodium citrate) concentration

3.6.1.1 To investigate the effect of sodium citrate stabilizer concentration on AgNPs stability, two concentrations of sodium citrate were used, i.e. 5 and 50 mM while the other synthesis conditions were similar to those stated in section 3.4.

3.6.1.2 Synthesized AgNPs stabilized with 5 mM sodium citrate were diluted to 0.2 mM. Five mL of each standard of GABA solution of 0, 100, 200, 300 and 400 mg L^{-1} concentration was pipetted and mixed with 5 mL of 0.2 mM of AgNPs, then vortexed for 20 seconds. Its absorbance in the wavelength range of 300 - 600 nm was determined with a UV-vis spectrophotometer in 60 seconds after mixing.

3.6.1.3 The above step in 3.6.1.2 were repeated with AgNPs that were prepared with 50 mM sodium citrate.

3.6.2 The effect of the surrounding medium

Dependency of aggregation behavior of AgNPs on the type of surrounding medium was investigated. The investigated media included acetate buffer (pH 3.8), citrate buffer (pH 3.8), phosphate buffer (pH 3.8) and hydrochloric acid at the same concentration of 0.1 M.

3.6.2.1 Five mL of a standard of GABA solution at 300 mg L^{-1} concentration was mixed with 5 mL of 0.2 mM of AgNPs and vortexed for 20 seconds. Its absorbance in the wavelength range of 300 - 600 nm was determined with a UV-vis spectrophotometer in 60 seconds after mixing.

3.6.2.2 The above steps in 3.6.2.1 and 3.6.2.2 were repeated with the other surrounding media.

3.6.2.3 For the preparation of a blank, the procedural steps in 3.6.2.1 and 3.6.2.2 were repeated but with DI water instead of a standard GABA solution.

3.6.3 The effect of pH

The effect of pH was investigated. The investigated solution pHs of 0.1 M acetate buffer were 3.8, 4.6 and 5.6. These buffer solutions were used to prepare the standard GABA solutions.

3.6.3.1 After 5 mL of standard GABA solution at 150 mg L^{-1} concentration was pipetted and mixed with 5 mL of 0.2 mM of AgNPs then vortexed for 20 seconds. Its absorbance in the wavelength range of 300 - 600 nm was determined with a UV-vis spectrophotometer in 60 seconds after mixing.

3.6.3.2 The procedural step in 3.6.3.1 was repeated but with changed concentrations of the standard GABA solutions to 0 and 300 mg L^{-1} concentration in that order.

3.6.3.3 The above steps in 3.6.3.1 - 3.6.3.2 were repeated with 0.1 M acetate buffer at other specified pHs.

3.6.4 The effect of acetate buffer concentration

The effect of acetate buffer concentration was investigated. The concentration of pH 3.8 acetate buffer solution was varied as 0.05, 0.1, 0.2, 0.5, 0.75 and 1.0 M.

3.6.4.1 Five mL of standard GABA solution at 150 mg L^{-1} concentration was pipetted and mixed with 5 mL of 0.2 mM of AgNPs then vortexed for 20 seconds. Its absorbance at the wavelength of 390 nm was determined with a UV- vis spectrophotometer in 60 seconds after mixing.

3.6.4.2 The procedural step in 3.6.4.1 was repeated but with changed concentrations of the standard GABA solutions to 0 and 400 mg L^{-1} in that order.

3.6.4.3 The steps in 3.6.4.1 - 3.6.4.2 above were repeated with the other concentrations of acetate buffer.

3.6.5 The effect of AgNPs concentration

To obtain an optimal AgNPs concentration for GABA analysis, the following steps were carried out.

3.6.5.1 AgNPs suspensions at different concentrations were prepared by diluting stock 0.5 mM AgNPs suspension with DI water. AgNPs suspensions at the concentrations of 0.1, 0.2 and 0.3 mM were obtained.

3.6.5.2 Five mL of 100 mg L^{-1} standard GABA solution was pipetted and mixed with 5 mL of 0.1 mM of AgNPs then vortexed for 20 seconds. Its absorbance at the wavelength of 390 nm was determined with a UV-vis spectrophotometer in 60 seconds after mixing.

3.6.5.3 The procedural step in 3.6.5.2 was repeated but with changed concentrations of the standard GABA solutions to 0, 200, 300 and 400 mg L^{-1} in that order.

3.6.5.4 The steps in 3.6.5.2 - 3.6.5.3 above were repeated with the other concentrations of AgNPs.

3.6.6 The effect of reaction time

Finally, the effect of reaction time (time that AgNPs were allowed to react with GABA) on SPR was determined.

3.6.6.1 Five mL of standard of GABA solution of 200 was pipetted and mixed with 5 mL of 0.1 mM of AgNPs then vortexed for 20 seconds. Its absorbance at

the wavelength of 390 nm was determined with a UV-vis spectrophotometer in 60 seconds after mixing. Specifically, a measurement was made in every 30 seconds for a period of 5 minutes.

3.6.6.2 The procedural step in 3.6.6.1 was repeated but with changed concentrations of the standard GABA solutions to 0 and 400 mg L^{-1} in that order.

3.7 Sample preparation for colorimetric AgNPs method

GABA food supplement capsules were purchased from local drug stores in Thailand. GABA powder of 0.75 g (one capsule) was dissolved in 100 mL of DI water and stirred for 10 minutes. The solution was filtered through a Whatman No. 1 filter paper and then a 0.22 μm nylon membrane.

3.8 Evaluation of analytical performance of the colorimetric AgNPs method

In this section, steps in the evaluation of the method's performance are studied. The performance was measured in terms of linearity of calibration, limit of detection, limit of quantification, precision and accuracy.

3.8.1 Construction of GABA calibration curve

Under optimal conditions, a calibration curve was constructed by plotting SPR absorbances against corresponding concentrations of standard GABA solutions. The absorbance for each concentration was measured in triplicates, and the mean of the triplicates was used for plotting.

3.8.1.1 Five mL of standard GABA solution at 100 mg L^{-1} concentration was pipetted and mixed with 5 mL of 0.2 mM of AgNPs then vortexed for 20 seconds. Its absorbance at the wavelength of 390 nm was determined with a UV-vis spectrophotometer in 60 seconds after mixing.

3.8.1.2 The procedural step in 3.8.1.1 was repeated but with changed concentrations of the standard GABA solutions to 0, 200, 300, 400 and 500 mg L^{-1} in that order.

3.8.1.3 The calibration curve was constructed by plotting the SPR absorbances at 390 nm against the concentrations of GABA.

3.8.2 Limit of detection (LOD) and limit of quantification (LOQ)

Evaluation in terms of LOD and LOQ was done by measurement of the absorbance of blank for triplicates. The LOD and LOQ were calculated using equation (3.1) and (3.2) , respectively,

$$\text{LOD} = \frac{3\text{SD of signal}}{\text{slope}}, \tag{3.1}$$

$$\text{LOQ} = \frac{10\text{SD of signal}}{\text{slope}}, \tag{3.2}$$

where SD is the standard deviation of the blank signal,

Slope is a slope of the calibration curve.

3.8.3 Precision

Precision of the method was determined in terms of percentage relative standard deviation (% RSD). Measurements were performed in seven replicates on a GABA standard solution of 300 mg L^{-1}. Calculation of % RSD was done according to equation (3.3) below,

$$\%\ \text{RSD} = \frac{\text{SD}}{\bar{X}} \times 100, \tag{3.3}$$

where SD is the standard deviation of seven replicates,

$\bar{x}$ is the mean of seven replicates.

3.8.4 Accuracy

The accuracy of the colorimetric AgNPs method was determined in terms of % recovery. Calculation of % recovery was by equation 3.4,

$$\%\ \text{recovery} = \frac{[\text{spiked sample}] - [\text{sample}]}{[\text{standard}]} \times 100, \tag{3.4}$$

where [spiked sample] is the concentration of GABA found in sample that spiked with the standard of GABA solution of 100 and 200 mg L^{-1},

[sample] is the concentration of the GABA food supplement samples,

[standard] is the standard of GABA solution of 100 and 200 mg L^{-1},

the experiment was carried by following step below.

3.8.4.1 Measurement of GABA concentration in food supplement samples

1) Three milliliters of the sample were added to 3 mL of 0.5 M acetate buffer (pH 3.8) and the mixture was adjusted to 25 mL with DI water then incubated overnight.

2) Five mL of the mixture was pipetted to 5 mL of 0.2 mM of AgNPs then vortexed for 20 seconds. Its absorbance at the wavelength of 390 nm was determined with a UV-vis spectrophotometer in 60 seconds after mixing.

3.8.4.2 Measurement of GABA concentration in the spiked sample

1) Three milliliters of a sample were spiked with 1.25 and 2.5 mL of stock GABA solution (2000 mg L^{-1}). Then, 3 mL of 0.5 M acetate buffer (pH 3.8) was added to the spiked sample and adjusted to 25 mL with DI water then incubated overnight. (The final concentration of GABA standard solution in sample were 100 and 200 mg L^{-1}.)

2) Five mL of the spiked sample was pipetted and mixed with 5 mL of 0.2 mM of AgNPs then vortexed for 20 seconds. Its absorbance at the wavelength of 390 nm was determined with a UV-vis spectrophotometer in 60 seconds after mixing.

3.9 Validation of the method

The developed colorimetric AgNPs method was validated with spectrophotometric method [25].

Briefly, 1 mL of 100, 200, 300, 400 and 500 mg L^{-1} of GABA was mixed with 0.6 mL of borax buffer (pH 8) and 2 mL of 2-hydroxynaphthaldehyde derivatizing reagent (0.3 % w/v in methanol). The resulting solutions were heated in a water bath at 80 °C for 10 min and allowed to cool to room temperature. Their volumes were adjusted to 10 mL with methanol then their absorption spectra were recorded in the wavelength range of 300-600 nm. The results obtained from the developed colorimetric AgNPs method were compared with those from the spectrophotometric method [25] by a paired *t*-test.

Part 2 Light scattering method

The colorimetric method can be used effectively with colorless samples, but with samples that have some color, the color will interfere strongly with the colorimetric measurement. Therefore, we have developed a second-order light scattering (SOS) method and automated it with SIA. For the determination of GABA in food supplement samples, an external calibration method was used whereas for green tea sample that contained several compounds that could interfere with the analysis, a standard addition method was used to minimize this problem.

3.10 Sequential injection system

The sequential injection system for automated determination of GABA is shown in Figure 3.1. It consisted of a carrier (DI water), a syringe pump, a holding coil, a multi-selection valve, AgNPs, 0.5 M acetate buffer of pH 3.8, stock solution of GABA 500 mg L^{-1}, a mixing coil (MC) and a spectrofluorometer. Spectrofluorometer was used to measure light scattering. In this study, The SOS excitation wavelength (λ_{Ex}) was set at 300 nm and the SOS intensity was recorded at 600 nm ($\lambda_{SOS}=2\lambda_{Ex}$).

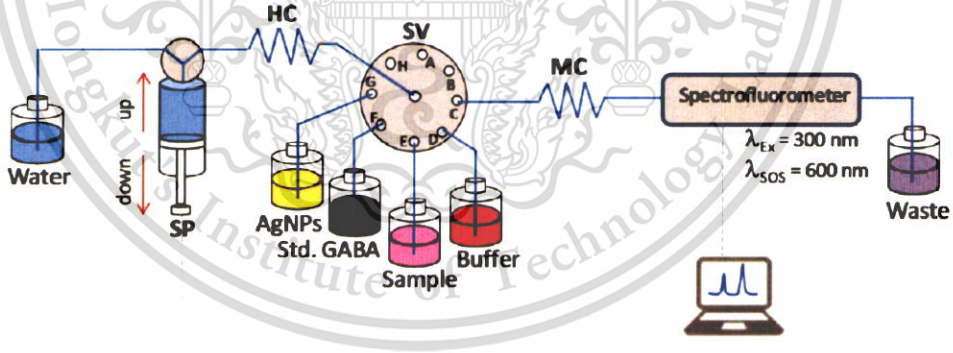


Figure 3.1 The configuration of the sequential injection system with second order light scattering for determination of GABA. SP: syringe pump, HC: holding coil (1 mm i.d. x 50 cm length), SV: eight-port selection valve, MC: mixing coil (1 mm i.d. x 50 cm length) and D: spectrofluorometer (λ_{Ex} = 300 nm, λ_{SOS} = 600 nm).

3.10.1 Steps of automated GABA determination by the sequential injection system

3.10.1.1 Aspirate 2,200 µL of the carrier into the syringe pump at a flow rate of 100 µL sec^{-1}.

3.10.1.2 Aspirate 80 µL of acetate buffer into the holding coil via port D.

Note: acetate buffer was used to adjust the volume of the GABA stock solution in order to achieve the desired concentration of GABA solution.

3.10.1.3 Aspirate 20 µL of the 500 mg L^{-1} GABA stock solution at a flow rate of 20 µL sec^{-1} into the holding coil via port F.

Note: in this case, the concentration of the GABA solution would be 100 mg L^{-1} in a total volume of 100 µL.

3.10.1.4 Aspirate 100 µL of the AgNPs suspension at a flow rate of 20 µL sec^{-1} into the holding coil via port G.

3.10.1.5 Transfer all of the solution zones in HC to the spectrofluorometer via port C at a flow rate of 20 µL sec^{-1} to measure the scattering value.

3.10.1.6 Repeat steps 1-5 but change the buffer volume to 70, 60, then 40 µL, 20 µL and the stock GABA solution to 30, 40, 60 then 80 µL in that order to achieve the desired GABA solutions at 150, 200, 300 and 400 mg L^{-1}, respectively. These volumes and concentrations are shown in table 3.3.

Table 3.3 The volumes of GABA stock solution (500 mg L^{-1}), 0.5 M acetate buffer (pH3.8) and AgNPs suspension that aspirated into HC.

Final concentration of GABA (ppm)	Volume of 500 mg L^{-1} GABA stock solution (µL)	Volume of 0.5 M acetate buffer (µL)	Volume of AgNPs suspension (µL)	Total volume (µL)
0	0.0	100.0	100.0	200.0
100	20.0	80.0	100.0	200.0
150	30.0	70.0	100.0	200.0
200	40.0	60.0	100.0	200.0
300	60.0	40.0	100.0	200.0
400	80.0	20.0	100.0	200.0

3.11 Optimization of light scattering method for GABA analysis.

Optimal conditions for GABA analysis with the SI system were investigated. The conditions included several physical and chemical parameters that affected the scattering intensity of AgNPs aggregates such as excitation wavelength, sequence zone, flow rate, mixing coil length, acetate buffer concentration, AgNPs concentration and AgNPs volume.

3.11.1 The effect of excitation wavelength

The effect of excitation wavelength on SOS scattering intensity was investigated. The investigated excitation wavelengths were 225, 250, 275, 300, 325 and 350 nm.

3.11.2 The effect of aspiration sequence

Aspiration sequence may affect the sensitivity of an analysis. Therefore, this parameter was studied and the different investigated aspiration sequence are shown in Figure 3.2. As can be seen in the Figure, the A sequence was non-sandwiched while the B sequence was sandwiched. The GABA zone was the zone of stock GABA solution at 500 mg L^{-1}; the AgNPs zone was the zone of AgNPs suspension at 0.2 mM; the buffer zone was the zone of acetate buffer at 0.5 M (pH 3.8). In the Figure, the volume ratio of acetate buffer + GABA stock solution to AgNPs suspension was 1:1. The final volume of AgNPs was 100 µL and the final volume of GABA standard solution with acetate buffer was also 100 µL.

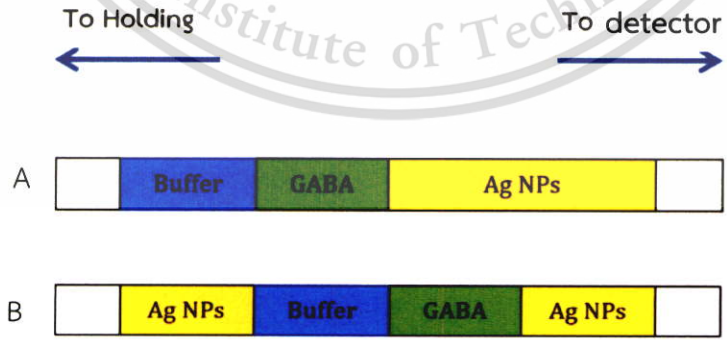


Figure 3.2 shows different aspirated zones investigated; the sequence A was non-sandwiched while the sequence B was sandwiched [39]

3.11.3 The effect of flow rate

The effects of flow rate on reaction time and sensitivity were investigated. The investigated aspiration/dispensing flow rates were 10/10, 15/15, 20/20 and 10/15 μl sec^{-1}.

3.11.4 The effect of mixing coil length

The effects of mixing coil length on reaction time and sensitivity were investigated. The investigated lengths were 0, 50, 100 and 150 cm.

3.11.5 The Effect of acetate buffer concentration

The effect of acetate buffer concentration on reaction time was investigated. The investigated acetate buffer concentrations were 0.01, 0.05, 0.1 and 0.5 M.

3.11.6 The Effect of AgNPs concentration

The effect of AgNPs concentration on reaction time was investigated. The investigated concentrations were 0.1, 0.2, 0.3, 0.4 and 0.5 mM.

3.12 Construction of GABA calibration curve

For the determination of GABA in the food supplement samples, an external calibration method was used while a standard addition method was used for the green tea sample. Once the optimal conditions for the automated GABA analysis with the SI system were obtained, a calibration curve was constructed by plotting the graph of the SOS scattering intensities versus the concentrations of the standard GABA solutions. Table 3.4 shows the operational steps of the SI system for the standard addition method.

Table 3.4 Operational sequences of sequential injection for determination of GABA by a standard addition method with in-line standard solution preparation.

Step	Direction of piston of SP	SV position	Action	Volume (μL)
1	down	-	aspirate DI water into syringe pump	2200
2	down	D	aspirate buffer solution into holding coil	varied[b]
3	down	E	aspirate sample containing GABA into holding coil	10
4	down	F	aspirate 500 mg L^{-1} of GABA standard solution into holding coil	varied[a]
5	down	G	aspirate AgNPs suspension into holding coil	100
6	up	C	dispense the mixture in holding coil into detector	2400

[a] The volumes of GABA stock solution were aspirated into the holding coil (0, 20, 30, 40, 60 and 80 μL).

[b] The volume of buffer was varied in order to adjust the total volume of it and GABA solution in the holding coil to 100 μL.

Note: For external calibration method, sample wasn't aspirated into the holding coil.

3.13 Samples preparation for light scattering method

Two kinds of food samples were analyzed: GABA food supplement powder in capsules and instant green tea powder.

3.13.1 GABA food supplement

GABA powder contained in a supplement capsule (~0.75 g) was weighed. Then, 100.0 mL of DI water was added to dissolve the powder. The solution was filtered through a Whatman No. 1 filter paper and then a 0.22 μm nylon membrane. An appropriate dilution (~50 times) was made by addition of a buffer to obtain a GABA concentration within the calibration range. The sample was clear and colorless.

3.13.2 Green tea powder

For the other kind of sample, green tea powder (3 g) was weighed and dissolved in 50.0 mL of hot DI water (~70 °C). The solution was centrifuged at 6000 rpm for 20 min. The supernatant was filtered through a Whatman No. 42 filter paper and a 0.22 μm nylon membrane. Then, 0.5 M acetate buffer (pH 3.8) was used to dilute the sample (~1.5 times) before the analysis. The green tea sample was pale yellowish-green in appearance.

3.14 Evaluation of the method's performance

In this section, steps in the evaluation of the method's performance are described. The performance was measured in terms of linearity of calibration, limit of detection, limit of quantification, precision and accuracy.

3.14.1 Construction of GABA calibration curve

Using the system in Figure 3.1 under optimal conditions, an external calibration curve was constructed by plotting the changes in SOS intensity (ΔI_{SOS}) against concentrations of standard GABA solutions at 100, 150, 200, 300 and 400 mg L^{-1}. The SOS intensity of standard GABA solution at each concentration was measured in triplicates, and the mean of the triplicates was used for plotting.

3.14.2 Limit of detection (LOD) and limit of quantification (LOQ)

Evaluation in terms of LOD and LOQ was done by measurement of the SOS intensity at GABA standard solution of 200 mg L^{-1} (five replicates). The LOD and LOQ were calculated by equation 3.1 and 3.2 where SD is the standard deviation of the signal for the 200 mg L^{-1} GABA standard solution and slope is the slope of the calibration curve.

3.14.3 Precision

The precision of the method was determined in terms of percentage relative standard deviation (%RSD) from five replicates of analysis of standard GABA solution at 200 mg L^{-1}. The precision was calculated by equation 3.3, where SD is the standard deviation of five replicates and $\bar{x}$ is the mean of five replicates.

3.14.4 Accuracy

Accuracy of the light scattering method was determined in terms of % recovery according to equation 3.4. In this study spiked sample is the concentration of GABA found in the sample that was spiked with a standard GABA solution of 100 mg L^{-1}. Sample is the concentration of the GABA food supplement sample or green tea sample. Standard is the standard GABA solution of 100 mg L^{-1}. The experiment was carried by following step below.

3.14.4.1 Measurement of GABA concentration in food samples

1) One milliliter of the sample was added to 1.5 mL of 0.5 M acetate buffer (pH 3.8) and the mixture was adjusted to 25 mL with DI water then incubated overnight.

2) The concentration of GABA was determined by SIA with SOS detection (The SOS excitation wavelength (λ_{Ex}) was set at 300 nm and the SOS intensity was recorded at 600 nm ($\lambda_{SOS}=_2\lambda_{Ex}$)).

3.14.4.2 Measurement of GABA concentration in the spiked sample

1) One milliliter of the sample was spiked with 10 mL of 500 mg L^{-1} stock GABA solution. Then 3 mL of 0.5 M acetate buffer (pH 3.8) was added to the spiked sample and adjusted to 50 mL with DI water then incubated overnight.

2) The concentration of GABA was determined by SIA with SOS detection. The % recovery of the spiked sample was then calculated.

3.15 Validation of the method

A high performance liquid chromatography (HPLC) method was employed for comparative purpose [25]. A 1.0 mL aliquot of GABA solution was added to 0.6 mL borax buffer (pH 8) and 1 mL of an 2-hydroxynaphthaldehyde derivatizing reagent (0.3 % w/v in methanol). The solution was heated in a water bath at 80 °C for 10 min and was allowed to cool. The final volume was adjusted to 5 mL with methanol. The final solution (5 mL) was injected into a Phenomenex C column (5 μm; 150 x 4.6 mm I.D.) and eluted with methanol: water (62: 38 v/v) at a flow-rate of 1 mL min^{-1}. The UV detector was set at 330 nm. For the HPLC method, a calibration curve in the range of 25-125 mg L^{-1} GABA was constructed. The results obtained from the SI method were compared with those from the HPLC method by a paired *t*-test.

Chapter IV

Results and Discussion

In this work, two methods of determination of Gamma-amino butyric acid (GABA) in food samples were developed based on silver nanoparticle sensor. The first method was a GABA detection based on absorption (colorimetric AgNPs). GABA was prepared with a net positive charge on its molecule so that it could induce negatively charged citrate capped AgNPs to aggregate. This resulted in a color change of the AgNPs suspension from yellow to green, which was spectrophotometrically monitored. This is the first report of the use of AgNPs for sensing GABA. The proposed method was used to detect GABA in a GABA food supplement. Subsequently, the second method was developed. It involved detection based on light scattering and some automation. Specifically, the developed system is a sequential injection system with second order scattering (SOS) detection for determination of GABA. The detection relied on positively charged GABA neutralizing negatively charged citrate-capped silver nanoparticles in acetate buffer. When the particle size increased, SOS intensity was enhanced. The concentration of GABA could be quantified by monitoring the relative change in SOS intensity using a spectrofluorometer. Unlike UV–vis detection, sample color posed no difficulty to analysis by SOS detection. The proposed method was applied to quantitation of GABA in food supplement and green tea powder.

4.1 Colorimetric AgNPs method for GABA analysis

The synthesized AgNPs were characterized by UV–visible spectrophotometry and transmission electron microscopy (TEM). The shape of the particles was roughly round with a diameter of 17 ± 4 nm. The color of the suspension was bright yellow. The maximum absorption was found to be at 390 nm, attributable to the SPR of monodispersed AgNPs. The monodispersion was a result of repulsion between

negative electrostatic layers of citrate caps on AgNPs, forcing each particle to remain separate from its neighboring particles. Reported in the literature [14, 29-30], a positively charged molecule could effectively trigger citrate-capped nanoparticles to aggregate. Accordingly, GABA could also induce particle aggregation if it had a net positive charge. Under this assumption, positively charged GABA was prepared and subsequently interacted with AgNPs.

GABA, a molecule containing carboxylic and amino groups, has an isoelectric point (pI) of 7.30. Therefore, a net positive electrical charge on the molecule can be generated when this molecule is dissolved in a medium with pH lower than this pI value. In the current study, GABA was prepared in acetate buffer with a pH of 3.8. After the introduction of the prepared GABA solution into the AgNPs suspension, the color of the suspension changed from yellow to green {from the aggregation of citrate-capped AgNPs of which the negative charges of the caps have been neutralized by positively-charged GABA. The SPR absorption of AgNPs decreased and a new shoulder was observed at approximately 500–600 nm (Figure 4.1). TEM images were used to verify the aggregation of AgNPs. TEM images of the AgNPs in the mixture are shown in Figure 4.1. As shown in the figure, TEM image of AgNPs with GABA in a acetate buffer is clearly different than that in a blank.

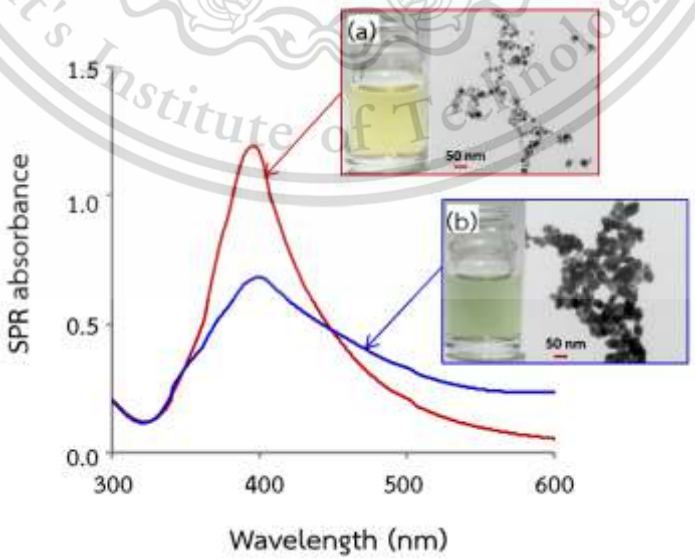
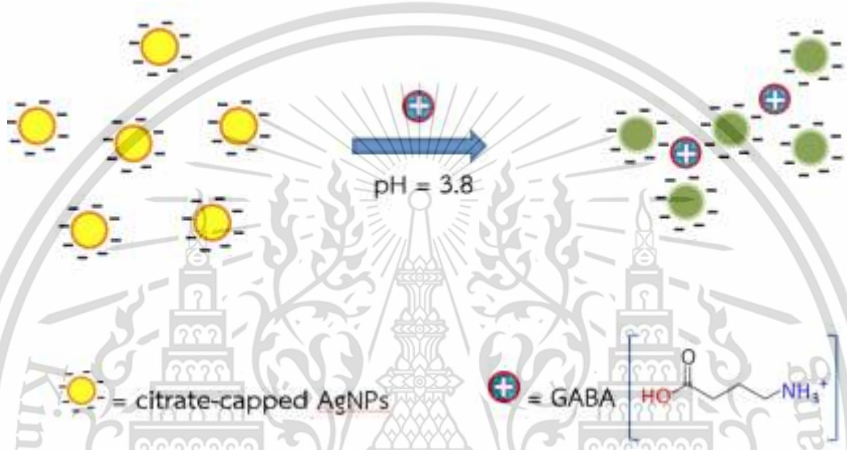


Figure. 4.1 SPR spectra, color and TEM images of AgNPs suspension added with acetate buffer (a) in the absence and (b) in the presence of 300 mg L^{-1} GABA.

For comparative purpose, GABA in DI water was prepared and added to AgNPs suspension. The pH of this GABA solution was 6.8, which implied that GABA was primarily in the form of zwitterion. This solution was dispensed into the AgNPs solution. As expected, the SPR intensity of AgNPs in the latter mixture did not change, confirming that the aggregation mechanism of AgNPs was caused by electrostatic interaction between negatively-charged citrate caps on AgNPs surface and the positive charges of GABA in acetate buffer. A schematic of the interaction is shown in schematic 4.1.



Schematic 4.1 Schematic illustration of the colorimetric detection of GABA based on citrate-capped silver nanoparticles.

4.1.1 Optimization of colorimetric AgNPs method for GABA analysis

4.1.1.1 The effect of stabilizer (sodium citrate) concentration

Early in the study, an AgNPs suspension was prepared following the method in [38], using 50 mM citrate as the capping agent. It was found that the resulting suspension was insensitive to GABA. This was probably due to too much negatively-charged citrate caps than even the highest 400 mg L^{-1} concentration of GABA was able to neutralize causing a greater degree of particle dispersion. Consequently, GABA was electrostatically unable to induce these particles to form clusters as shown in Figure 4.2 (a), the SPR absorbance of AgNPs suspension did not exhibit any changes with or without GABA. The AgNPs preparation was then

later modified to use 5 mM of citrate as a stabilizer instead of 50 mM. Under this new condition, the resulting AgNPs suspension showed a superior capability to detect GABA (Figure 4.2 (b)). The zeta potentials of AgNPs obtained by these two different preparation protocols were determined using a zeta analyzer. The zeta potentials of the AgNPs stabilized by 5 and 50 mM of citrate were −0.118 and −0.260 mV, respectively. This indicated that excessive amounts of citrate afforded a larger number of negative ions on the particles' surface which resulted in too well-stabilized particles that were difficult to aggregate in the presence of GABA. Hence in this work, 5 mM of citrate was used as a suitable capping agent preparing AgNPs suspension for GABA determination.

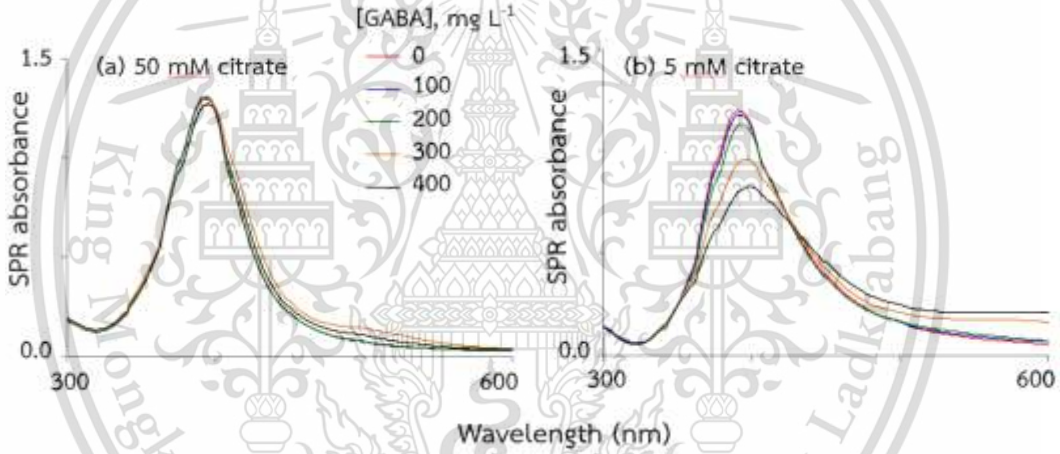


Figure 4.2 Spectra of AgNP suspensions in the presence of 0, 100, 200, 300 and 400 mg L^{-1} GABA, prepared by using citrate concentrations of (a) 50 mM and (b) 5 mM.

4.1.1.2 Effect of surrounding media

The effect of the surrounding media on the reaction between GABA and AgNPs was investigated. The media under study included acetate, citrate and phosphate buffers as well as hydrochloric acid. The SPR spectra of AgNP suspensions in different media before and after addition of GABA were compared. The SPR absorption peak of AgNPs suspended in acetate buffer declined after an

addition of GABA (Figure 4.1), whilst in citrate and phosphate buffers, the SPR absorption either with and without GABA was almost unchanged, indicating that these media did not facilitate particle aggregation (Figure 4.3 (a) and (b)). Moreover, the particles were readily aggregated in hydrochloric acid, even in the absence of GABA (Figure 4.3 (c)). Accordingly, acetate buffer was selected as the most suitable medium.

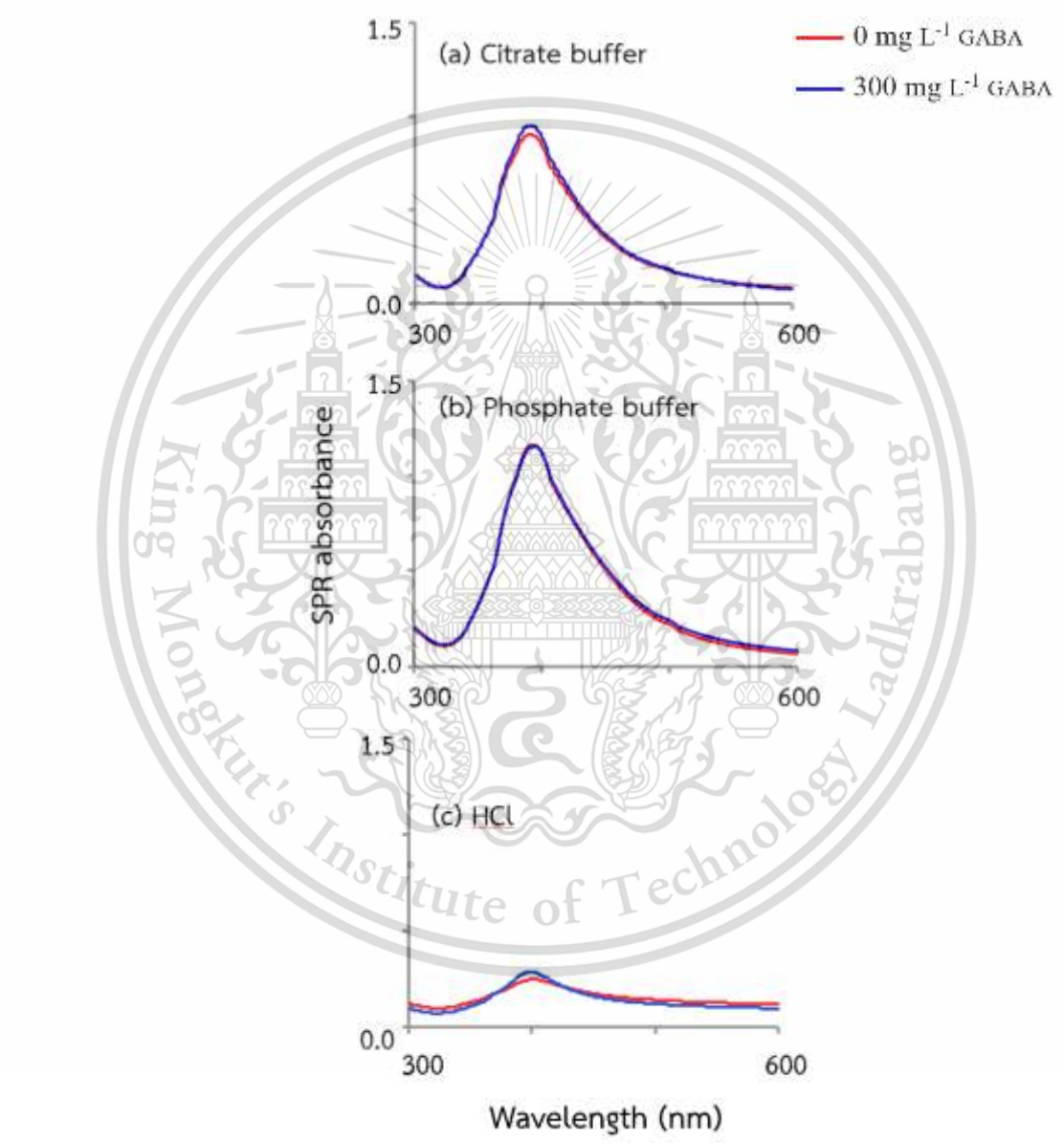


Figure 4.3 SPR spectra of AgNP solutions in the absence and presence of 300 mg L^{-1} of GABA in media consisting of (a) citrate and (b) phosphate buffer, and (c) HCl.

4.1.1.3 Effect of pH

The pH of a medium not only influenced the electrical charge of the target analyte, but also the particle stability [14]. In this experiment, various pH values that were less than the pI of GABA were tested. Standard solutions of GABA were prepared in acetate buffer at pH values of 3.8, 4.6 and 5.6. These solutions were added to the AgNPs suspension. It was found that the medium with the pH value of 3.8 provided a better response for detection of GABA than the other pH values. Since citrate was a weak acid with three pK_a values at 3.1, 4.7 and 6.4, the solution pH affected the charge state of its ion. At higher pH values (4.6 and 5.6), carboxylates of citrate tended to be ionized, resulting in negatively charged particle surface. A negative charged particle surface promoted extensive repulsion between particles. The electrostatic interaction between the positively charged GABA and negatively charged particles might be insufficient to overcome the repulsion forces among particles; therefore, aggregation of AgNPs was not attained. The SPR absorbance did not change, supporting the data in Figure 4.4. However, at pH 3.8, carboxylates were more protonated, decreasing the negative charges on the particle surface. The interparticle distance was shortened. When GABA was introduced into this suspension, the particles rapidly aggregated. As a result, the SPR intensity gradually declined with the concentration of GABA (Figure 4.4). In order to confirm the above phenomenon, the zeta potentials of particles at different pH values were measured. It was found that the zeta potential of AgNPs dispersed in a suspension with a pH value of 3.8, 4.6 and 5.6 were -0.019, -0.067 and -0.083 mV, respectively. These results reflected that at a higher pH value, more ionized citrate ions were obtained, leading to a higher degree of dispersion of particles with less sensitivity to GABA.

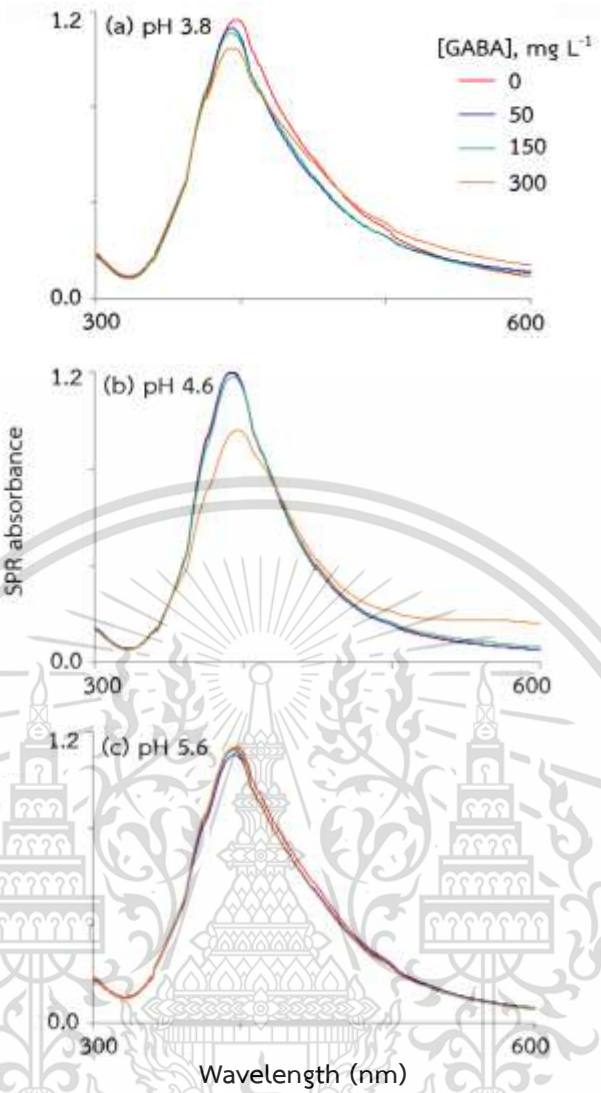


Figure 4.4 SPR spectra of AgNPs suspensions upon addition of GABA at various concentrations; GABA in a 0.1 M acetate buffer at pH (a) 3.8, (b) 4.6 and (c) 5.6.

4.1.1.4 Effect of acetate buffer concentration

Acetate buffer concentration affected the aggregation process. Typically, the repulsion of negatively charged nanoparticles could deteriorate in a high acetate buffer concentration environment due to electrostatic shielding effect [40]; consequently, self-aggregation of particles could occur even in the absence of a positively-charged analyte. With this in mind, the ionic strength was varied in this experiment by varying the concentration of the acetate buffer from 0.05 to 1.0 M. In

Figure 4.5 (a), when 0.05 M buffer was used, the AgNPs did not aggregate when GABA at the concentrations of 150 and 400 mg L^{-1} were added. On the other hand, AgNPs aggregation was more easily induced by GABA when the buffer concentration was increased to 0.5 M. This lead to a decrease in SPR intensity at 390 nm after the addition of GABA. However, at buffer concentrations higher than 0.5 M, some agglomeration was found even though GABA had not even been introduced. Hence 0.5 M acetate buffer (pH 3.8) was employed for the current study.

4.1.1.5 Effect of concentration of AgNPs

A further investigation focused on the concentration of AgNPs was performed. In this work, the as-prepared AgNPs concentration (calculated based on the final concentration of $AgNO_3$) was 0.5 mM. This suspension was diluted to 0.1-0.3 mM prior to reaction with GABA. The results showed that 0.1 mM of AgNPs provided a poor detection of GABA, while 0.2 and 0.3 mM provided a sensitivity (Figure 4.5 (b)); therefore, in order to minimize reagent consumption, 0.2 mM of AgNP suspension was chosen for the assay.

4.1.1.6 Effect of reaction time

The reaction time of the proposed method was determined by monitoring the decrease in the SPR absorbance as a function of time after the AgNPs suspension was mixed with GABA. As shown in Figure 4.5 (c), the absorbance decreased noticeably in 1 min. Then, the absorbance gradually declined and remained constant after 3 min. Since rapid analysis was desirable, a detection time of 1 min was thus chosen.

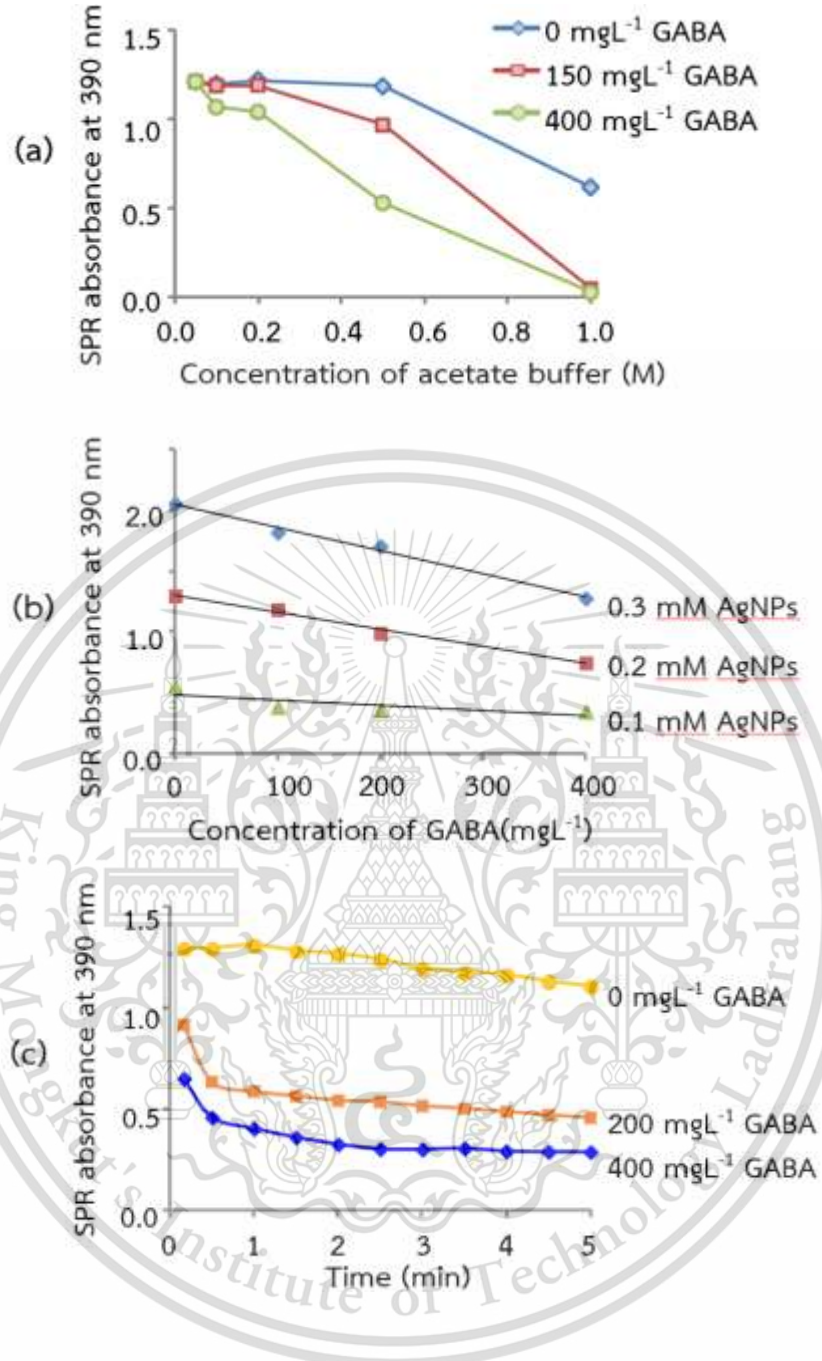


Figure 4.5 Effects of (a) ionic strength, (b) concentration of AgNPs and (c) reaction time on the SPR absorbance of the AgNPs-GABA interaction.

4.1.2 Interference study

The influence of foreign species that were sometimes found in formulations of GABA food supplements was investigated. These included inorganic cations (Na^+, K^+ and Mg^{2+}) and organic compounds (glucose, fructose, glycine and

vitamin B_6). At the concentration of 300 mg L^{-1}, all species tested including GABA were individually exposed to the AgNP sensor. As shown in Figure 4.6, of all of the species examined, only Mg^{2+} and GABA caused obvious changes in the SPR absorbance of AgNPs. This result indicated that Mg^{2+} could interfere with the assay. However, the amount of Mg^{2+} usually found in these supplements was very low (e.g., 62.5 mg Mg^{2+}/500 mg GABA capsule). With the sample preparation described in Section 3.7, the final concentration of Mg^{2+} in the samples was determined to be 7.5 mg L^{-1}. Therefore, in the next step, 7.5 mg L^{-1} of Mg^{2+} was prepared and spiked into the AgNPs suspension. It was found that Mg^{2+} at this level could be tolerated and did not influence the determination of GABA significantly. Hence, the AgNP sensor had a good selectivity and could potentially be applied to determination of GABA in food supplements.

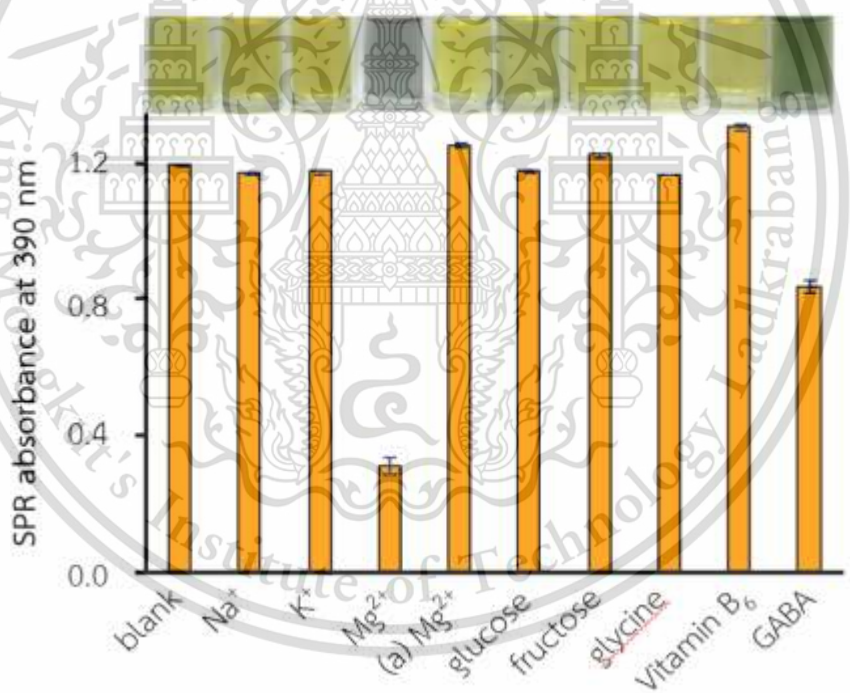


Figure 4.6 SPR absorbance of AgNP sensor with the addition of 300 mg L^{-1} of several ions/compounds; the (a) Mg^{2+} was at a concentration of 7.5 mg L^{-1}; the inset shows images of the mixtures with different colors that corresponded to the particular ions/compounds tested.

4.1.3 Analytical performance of AgNPs sensing of GABA

Under optimal conditions, AgNPs were used as a colorimetric probe for quantitative determination of GABA. It can be seen in Figure 4.7 that the color of AgNPs suspension changed from yellow to green with increasing concentration of GABA from 0–500 mg L^{-1}. Correspondingly, the SPR intensity of AgNPs suspension at 390 nm decreased, and the decreased intensity was accompanied by broader peaks with a slight red shift. Simultaneously, a shoulder at approximately 500–600 nm emerged. A calibration curve was constructed by plotting the change in absorbance (Abs) at the wavelength of maximum absorption versus the GABA concentration. The curve was linear over the range of 100–500 mg L^{-1} (Figure 4.8). A regression was done and the limit of detection (LOD) and quantification (LOQ) (determined from 3 and 10 times the standard deviation of the blank, respectively) were found to be 57.7 and 79.2 mg L^{-1} of GABA, respectively. Although the detection limit of the developed method was higher than that of the HPLC method [3,25], it was sensitive enough for determination of GABA levels in food supplements. The reproducibility of the method was evaluated based on the relative standard deviation (RSD) of seven determination replicates of 300 mg L^{-1} of GABA. An RSD of 3.2% was obtained, indicating that the method provided a good reproducibility of measurement.

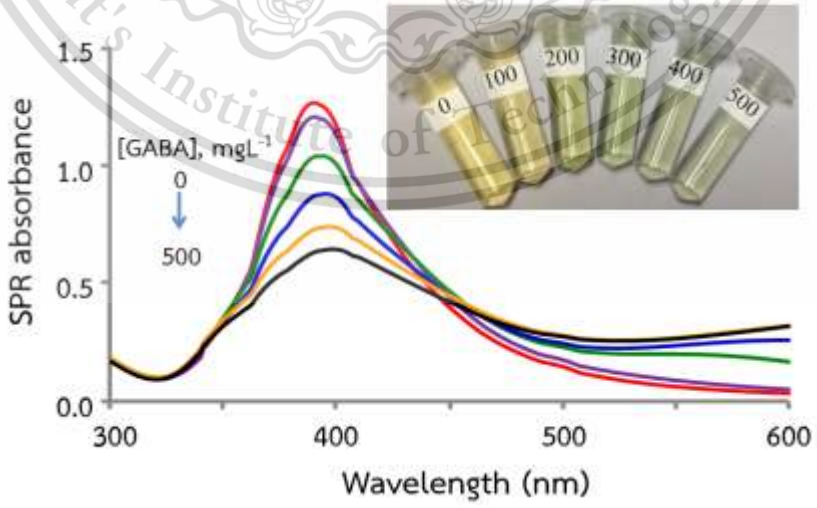


Figure 4.7 SPR spectra and photos of AgNPs suspensions added with GABA at various concentrations of GABA.

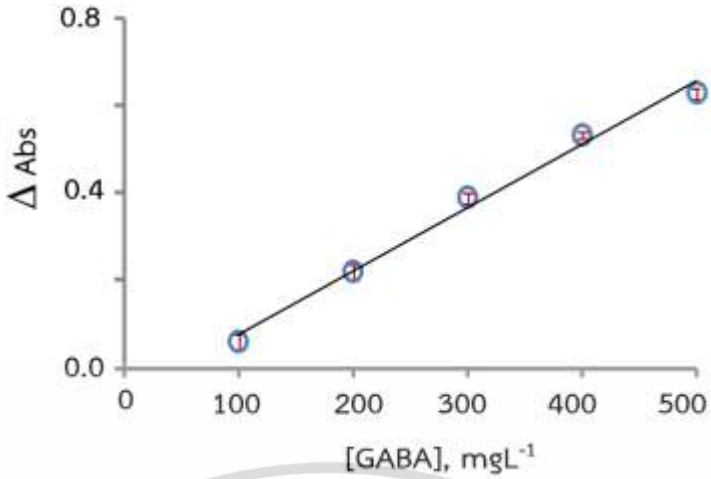


Figure 4.8 A linear relationship between the change of SPR absorbance (ΔAbs) and GABA concentration

4.1.4 Analysis of food supplement samples

To evaluate the feasibility of using the developed assay for determining GABA content in food supplements, analytical recoveries were investigated. Samples were prepared by spiking them with GABA at various concentrations. The results of this analysis are shown in Table 1. The recoveries ranged from 97.5 to 104.5%, indicating that the sample matrices did not interfere with this determination method.

Table 4.1 Recovery results of GABA in food supplements using the developed AgNP sensor.

Sample	GABA concentration (mg L^{-1})			Recovery (%)
	Detected	Added	Found	
A	110.0±4.6	100.0	210.7±3.4	100.7
B	118.5±2.2	200.0	327.5±6.9	104.5
C	131.8±3.4	100.0	236.3±3.2	104.5
D	221.0±6.4	100.0	318.5±4.4	97.5

For validation purpose, four food supplement samples were analyzed by using both the developed method and a spectrophotometric method based on 2-hydroxynaphthaldehyde derivatization [5]. The analysis results are shown in Table 4.2. They were statistically compared by a paired t-test. There was no significant difference between the results obtained from these two methods at a 95% confidence level ($t_{observed}$ = 0.57, $t_{critical}$ = 4.30). This showed that our new method for measuring GABA in food supplement was reliable. Compared to conventional HPLC method [3,25], the developed method could be performed at room temperature without the use of a derivatizing agent. Moreover, the reaction proceeded quickly, requiring only 1 min per measurement. The method was inexpensive and more practical for routine use than an enzyme assay [27]. Unlike other sensors reported in the literature [27,28], this colorimetric AgNP probe is simple. It does not involve complicated electrode preparation and does not require high level of technical skill.

Table 4.2 Comparison of the colorimetric AgNP sensor for quantification of GABA and a 2- hydroxynaphthaldehyde method.

Sample	GABA content (mg per capsule ± confidence intervals from three replicates)		
	labelled	AgNPs sensor	2-Hydronaphthaldehydemethod [25]
A	750	742.8±5.6	744.3±7.2
B	750	727.2±4.3	732.4±1.6
C	500	498.8±6.8	497.3±5.1
D	500	504.5±4.1	491.9±2.1

4.2 Light scattering method for GABA analysis

In this part, a determination of GABA by reaction with silver nanoparticles was developed in combination with a sequential injection system to automate the determination. Positively- charged GABA induces aggregation of negatively- charged

citrate-capped AgNPs. The aggregation of AgNPs into bigger clusters causes changes in light scattering which can be measured by spectrofluorometer. The higher the degree of aggregation, the higher the scattering intensity. Based on these principles, optimal conditions for determining GABA using SI with second order light scattering (SOS) detection were investigated. The intensity of the SOS was detected at the corresponding double wavelength of excitation, i.e. $\lambda_{SOS} = 2\lambda_{Ex}$.

4.2.1 Optimization of light scattering method for GABA analysis

4.2.1.1 The effect of excitation wavelength

An investigation of the effect of excitation wavelength (λ_{Ex}) on the sensitivity of GABA analysis was performed. The investigated wavelengths were 225, 250, 275, 300, 325 and 350 nm and the investigated GABA concentrations at 400 mg L^{-1} shown in Figure 4.9. At these excitation wavelengths, it was found that the maximum ΔI_{LS} was at the SOS wavelength (λ_{SOS}) of 450, 500, 550, 600, 650 and 700 nm, respectively.

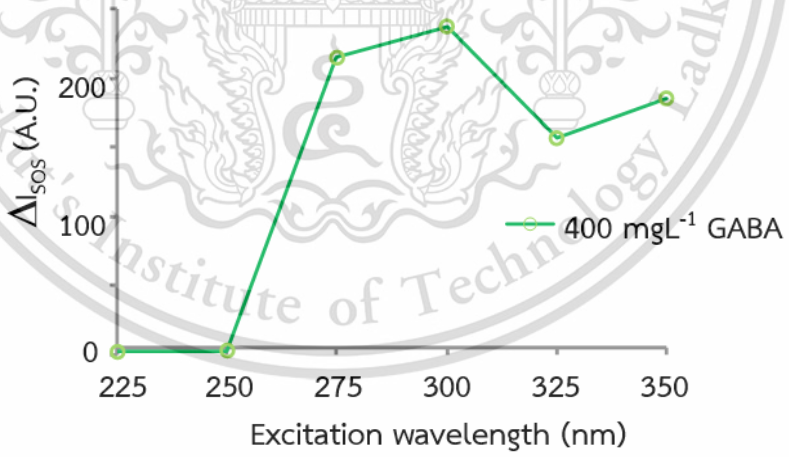


Figure 4.9 Plots of the difference between the scattering intensity of the 0.4 mM AgNPs solution interacted with 0 and 400 mg L^{-1} standard GABA solution and the blank (ΔI_{LS}) versus the excitation wavelength of 225, 250, 275, 300, 325 and 350 nm.

The results of the investigation showed that λ_{Ex} of 300 nm and λ_{sos} 600 nm yielded the highest ΔI_{LS} for 0 and 400 mg L^{-1} GABA solution, hence this λ_{Ex} was selected as the optimal excitation wavelength in this study.

4.2.1.2 Effects of aspiration sequence

The effect of aspiration sequence on the sensitivity of the determination procedure was investigated, and sandwiched and non- sandwiched sequences were compared. The aspiration sequence that yielded the highest determination sensitivity was then selected. The results are shown in Table 4.3.

Table 4.3 Linear equations and correlation coefficients (R^2) obtained when using different aspiration sequences

aspiration sequence	Linear equation	R^2
Buffer/ Std.GABA/ AgNPs	y = 0.5593x + 58.119	0.9237
AgNPs/ Buffer/ Std.GABA/ AgNPs	y = 0.2894x + 0.6737	0.7293

The results show that the buffer/Std.GABA/AgNPs was the best aspiration sequence in terms of R^2 (0.9237). Hence, buffer/ Std.GABA/ AgNPs was the aspiration sequence chosen for the final protocol.

4.2.1.3 The effect of flow rate

In this study, aspiration and dispensing rates were kept to be the same. The aspiration and dispensing rates investigated were 10/ 10, 15/ 15 and 20/ 20 μL sec^{-1}. The optimization results are shown in Table 4.4.

Table 4.4 Linear equations and correlation coefficients obtained by using the SIA with different aspiration and dispensing rates.

Flow rate (aspiration/dispensing) (μL sec^{-1})	Linear equation	R^2	Sample throughput (samples/hr)
10/10	y = 0.4143x + 14	0.9991	8
15/15	y = 0.2957x + 2	0.9643	11
20/20	y = 0.3971x + 28	0.9725	14

The results show that the aspiration/dispensing rates of 10/10 μL sec^{-1} provided the best sensitivity, but the sample throughput was low; therefore, the flow rate of 20/20 was selected instead because of its higher throughput with an adequate sensitivity.

4.2.1.4 The effect of mixing coil length

An investigation was performed on the effect of mixing coil length on the sensitivity of GABA analysis. The lengths investigated were 0, 50, 100 and 150 cm. The results are shown in Table 4.5.

Table 4.5 Linear equations and correlation coefficients obtained from using the SIA with different mixing coil lengths.

Mixing coil length (cm)	**Linear equation**	R^2	**Sample throughput (samples/hr)**
0	y = 1.5447x + 75.243	0.698	13
50	y = 0.8734x + 90.959	0.959	14
100	y = 1.0973x + 27.432	0.939	13
150	y = 0.5827x + 86.568	0.923	13

As the mixing coil length increased, the distance along the coil length that the reaction took place increased hence the reaction time between the standard GABA solution and AgNPs suspension increased. The results showed that the 50 cm coil length provided sufficient sensitive with good R^2 and sample throughput. So the optimal coil length 50 cm was chosen.

4.2.1.5 The effect of acetate buffer concentration

An investigation was performed on the effect of pH 3.8 acetate buffer concentration on the interaction between AgNPs and GABA. The buffer concentrations investigated were 0.01, 0.05, 0.1, 0.5 and 1 M. Buffers at these concentrations were used in the preparation of 0, 200 and 400 mg L^{-1}standard GABA solutions and in volume adjustment in the SIA system. The results are shown in Figure 4.10

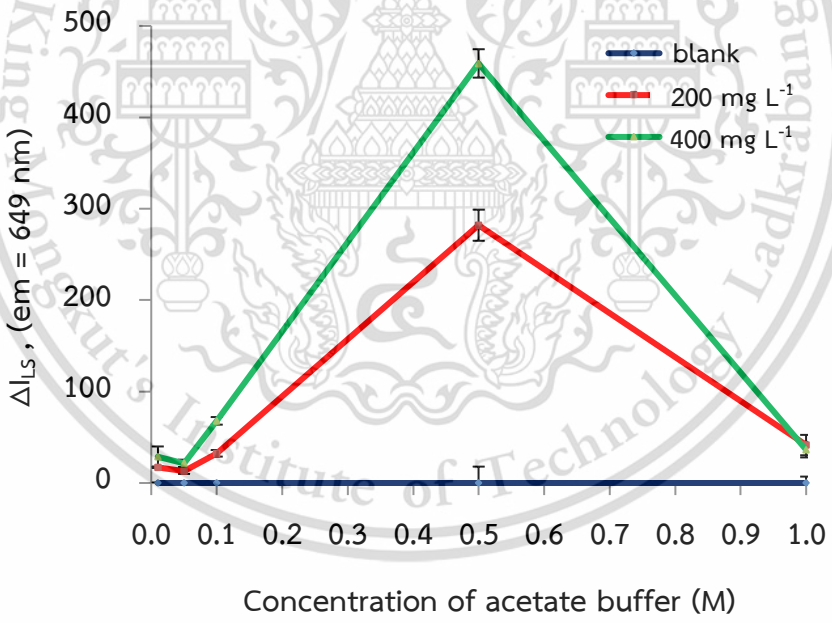


Figure 4.10 Plots of the difference between the scattering intensity of the standard GABA solution and the blank (ΔI_{LS}) versus acetate buffer concentration.

Buffer concentration dictates the ionic strength of the standard GABA solutions and the volume to be adjusted by the SIA system. It can be seen in

Figure 4.10 that for a low buffer concentration (0.01-0.1 M) in the mixture, GABA was not able to induce aggregation of AgNPs well, hence the ΔI_{LS} was low. For a higher buffer concentration (0.5 M), the ionic strength was optimal for inducing good AgNPs aggregation; therefore, when GABA at various concentrations were added to AgNPs suspension, the aggregation was more complete, giving a high value of ΔI_{LS}. However, for a higher buffer concentration (1 M), self-aggregation of AgNPs occurred from high ionic strength [16], so ΔI_{LS} did not vary linearly with GABA concentration. Hence, the concentration of acetate buffer of 0.5 M was selected as the optimal concentration in this study.

4.2.1.6 The effect of AgNPs suspension concentration

An investigation of the effect of AgNPs suspension concentration on the sensitivity of GABA analysis was performed. The investigated concentrations were 0.2, 0.3, 0.4 and 0.5 mM. The suspensions at these concentrations were used to react with 0, 200 and 400 mg L^{-1}. The results are shown in Figure 4.11.

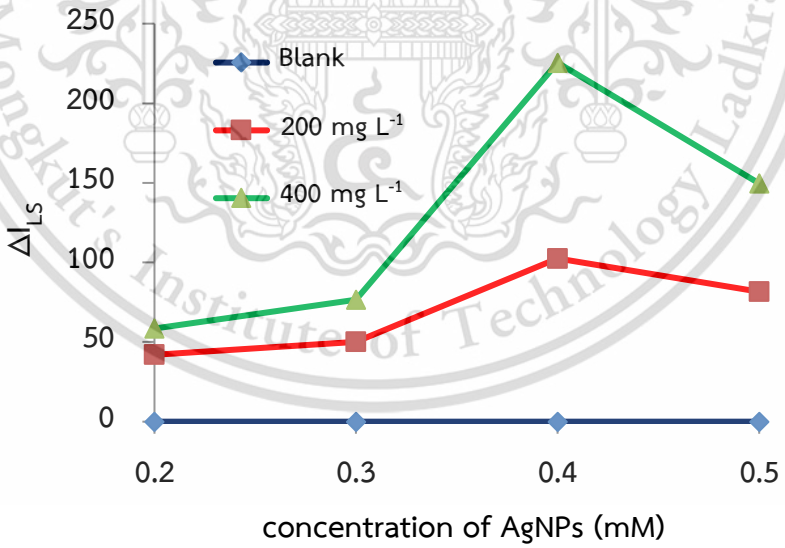


Figure 4.11 Plots of the difference between the scattering intensity of the standard GABA solution and the blank versus AgNP concentrations.

The results showed that the highest difference between the intensities of the GABA standard solution and the blank was obtained with the AgNPs suspension at 0.4 mM. This might be because at 0.2 and 0.3 mM, there were fewer numbers of AgNPs to get aggregated and scatterd light, while at 0.5 mM, there were too much AgNPs that blocked the scattered light from reaching the detector. Therefore, 0.4 mM was selected as the optimal concentration of AgNPs suspension for this method of GABA analysis.

4.2.2 Calibration curve of standard GABA solutions analyzed under optimal conditions

Under the optimal conditions, an analysis of standard GABA solutions with AgNPs and SI system was performed, and the signals from the procedure, as shown in Figure 4.12 were used to construct a calibration curve of standard GABA solutions shown in Figure 4.13

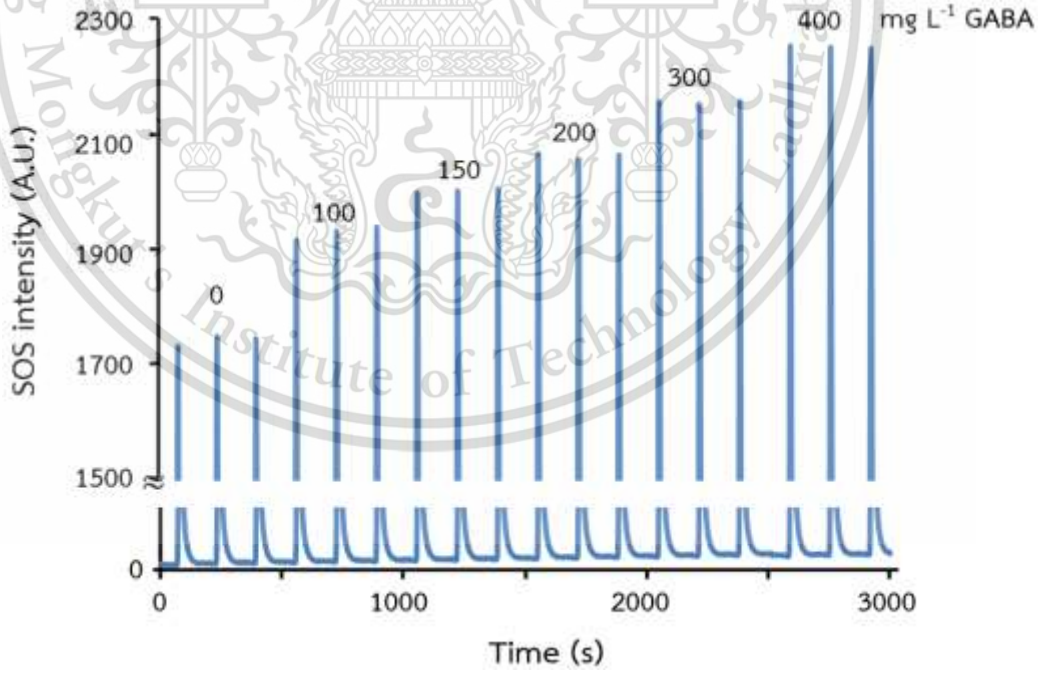


Figure 4.12 Examples of signal profiles obtained by the sequential injection in Figure. 4.13 for external calibration.

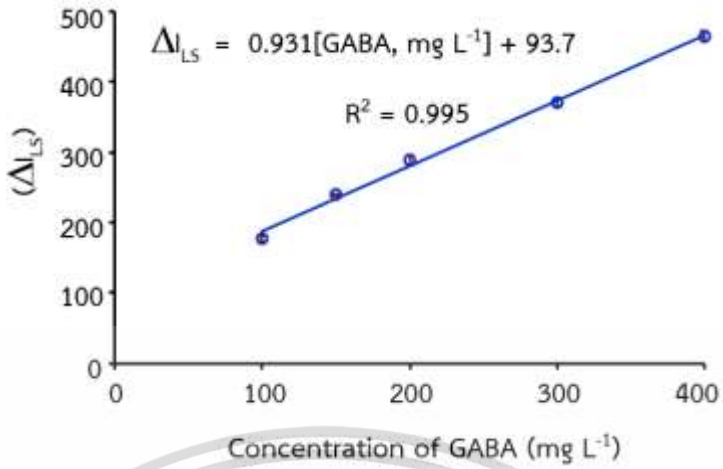


Figure 4.13 Calibration curve of standard GABA solutions in the range of 100-400 mg L^{-1}

4.2.3 Performance evaluation of the developed method

Using the system in Figure 3.1 under optimized conditions, well- defined signal profiles were achieved (Figure 4. 11). External calibration was performed and a calibration curve was constructed, plotting the change in SOS intensity (ΔI_{SOS}) against GABA concentration. A linear relationship was found for the concentration range of 100- 400 mg L^{-1} GABA. The regression equation was ΔI_{SOS} = (0.931±0.035)[GABA,mg L^{-1}] + (93.7±9.08), with a regression coefficient (R^2) = 0.995. The limit of detection (LOD) and limit of quantitation (LOQ) were defined as 3 and 10 times the standard deviation of the signal generated by 200 mg L^{-1} GABA (5 replicates) and found to be 39. 6 and 132.0 mg L^{-1} GABA, respectively. The reproducibility of the method was evaluated from the relative standard deviation (RSD) value [41] at 200 mg L^{-1} GABA signal (5 replicates). A % RSD of 0. 6% was obtained, indicating that the method provided good reproducibility of measurements. The throughput of the analytical method was found to be 25 h^{-1} with an external calibration. A standard addition method was carried out using the same concentration range for standard solutions as that for the external

calibration. The R^2 of each individual standard addition line was >0.99. Acceptable precision of analysis was achieved (RSD = 3.3%, 3 replicates).

4.2.4 Determination of GABA content in samples

The developed method was applied to the determination of GABA in food supplement and instant green tea samples. In the supplement, GABA was a major component of the formulation, and thus the sample was categorized as a low matrix sample. An external calibration was employed for the analysis. The green tea samples contained many ingredients, and thus standard addition method was employed. The analytical results are summarized in Table 4.6. A good agreement between the two sets of results was attained at 95% confidence level ($t_{observed}$ = 0.96, $t_{critical}$ = 4.30). Recoveries were determined by spiking 100 mg L^{-1} GABA into the samples prior to analysis. Satisfactory recoveries were obtained in the range of 93.1–97.5% for the supplement samples and 99.4–102.2% for green teas samples. The main advantage of this method is that it can be applied for quantification of GABA in colored food samples. This SI method is rapid, automated and reliable. Moreover, the analysis consumes low volume of sample and AgNP reagent which reduces waste production.

Table 4.6 Comparison of GABA contents determined by the developed sequential injection (SI) method versus an established HPLC method.

Sample	Analytical approach	GABA (mg) per capsule or sachet		
		Labelled	SI method	HPLC method
food supplement 1	External calibration	750	771.3±7.1	781.5±10.2
food supplement 1	External calibration	750	753.4±4.5	736.4±2.5
Instant green tea 1	Standard addition	20	19.6±6.9	21.1±1.9
Instant green tea 1	Standard addition	20	25.1±2.3	21.0±2.4

Chapter V

CONCLUSION

This work was a development of a gamma-aminobutyric acid (GABA) assay with silver nanoparticles (AgNPs). The AgNPs were synthesized by using $NaBH_4$ as a reducing agent and sodium citrate at 5 mM as a stabilizer. The synthesized AgNPs were characterized by transmission electron microscopy and UV–visible spectrophotometry. It was found that the nanoparticles were spherically shaped with a diameter of 17 ± 4 nm. The suspension was bright yellow in color. The maximum absorption peak was found to be 390 nm, and the peak was attributed to surface plasmon resonance (SPR) of monodispersed AgNPs. Monodispersion was due to repulsion between the negative electrostatic layers of citrate caps on the surface of neighboring AgNPs, forcing each particle to stay separate from its neighboring particles. The GABA to be determined was prepared to posses a positive charge on its molecule so that it could neutralize these negative electrostatic layers and induce AgNPs to aggregate. The aggregation caused the color of the AgNPs suspension to change from yellow to green, which can be detected by a spectrophotometric method. The whole procedure was facile and low-cost. The reaction could be made at room temperature and required only 1 min for complete detection. The calibration curve was linear over the range of 100–500 mg L^{-1}, and the limits of detection (LOD) and quantification (LOQ) were 57.7 and 79.2 mg L^{-1} of GABA, respectively. The percentage recoveries achieved ranged from 97.5 to 104.5 %, indicating that the sample matrix did not interfere with the detection by this method. For validation purpose, four GABA food supplement samples were analyzed by both the developed method and an established spectrophotometric method that employed 2-hydroxynaphthaldehyde derivatization. The results were statistically compared by a paired t-test. It was found that

there was no significant difference between the results obtained from these two methods at 95% confidence level (tobserved = 0.57, tcritical = 4.30). This shows that our developed method for measuring GABA in food supplement is reliable.

Light scattering method, a sequential injection (SI) method with second order light scattering (SOS) was developed for automated determination of GABA in food products. The SI system allows automated in-line preparation of working standard solutions, which significantly reduces manual labor. In the determination procedure, GABA induces AgNPs to aggregate causing an increase in the intensity of SOS. The SOS intensity increases proportionally with the concentration of GABA and is not interfered by strong color. The use of light scattering detection has a potential to successfully determine GABA in colored samples. The developed method is reproducible, rapid and sensitive. The LOD and LOQ achieved by this developed method were 39.6 and 132.0 mg L^{-1} GABA, respectively. A %RSD of 0.6% was obtained, indicating that the method provides a good reproducibility of measurements. The throughput of this analysis method was found to be 25 h^{-1}. Satisfactory recoveries were obtained in the range of 93.1–97.5% for GABA in food supplement samples and 99.4–102.2% for GABA in green tea samples. The advantage of this method is that it can be applied for automated quantification of GABA in colored food samples.

Suggestions for future work

Other types of samples should be investigated. The selectivity of the analytical method should be further refined in order for it to be able to analyze trace of GABA in food samples with many other chemical components.

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Appendices

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A novel colorimetric method for detection of gamma-aminobutyric acid based on silver nanoparticles

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ABSTRACT

A novel, simple and rapid colorimetric method for determination of gamma-aminobutyric acid (GABA) was developed using a negatively charged citrate-capped silver nanoparticle (AgNP) probe. At an acidic pH of 3.8, GABA had a positive charge due to a protonation of amine groups. Therefore GABA could induce an aggregation of AgNPs due to electrostatic interaction. This caused a decrease in the surface plasmon resonance spectra of the colloidal solution at wavelength of 390 nm with a slight red shift. At the same time, the color of the suspension turned from yellow to green upon aggregation. Quantification of GABA was done spectrophotometrically. Under optimum conditions, the method showed a linear calibration in range of 100–500 mg L^{-1} GABA, with a detection limit of 57.7 mg L^{-1}. The method was successfully applied to measure GABA quantities in dietary supplements.

1. Introduction

Nowadays, consumers pay much attention to healthy lifestyles, so that they have become increasingly interested in consuming natural, nutritional and healthy foods. Following this trend, many food manufacturers improved their product quality by fortifying their products with nutrients and vitamins.

Gamma-aminobutyric acid (GABA) is a nutritional compound that has received great attention from consumers over the past decade. GABA is a non-protein amino acid that is a predominant neurotransmitter in the central nervous system of mammals. It can lower blood pressure, induce a tranquilizing effect and improve brain function [1]. Recently, it has been reported that GABA may have potential for treatment of Alzheimer's disease [2]. This compound also has an inhibitory action against the proliferation of cancer cells [3]. In some Asian countries, including Japan and Thailand, extracted GABA is commonly added to foods and beverages to convey a sense of healthfulness as a way to increase consumer interest and add value to their products. Various brands of GABA dietary supplements are available in the market.

High performance liquid chromatography (HPLC) is a technique widely used for evaluating GABA levels in foods. However, direct detection of this compound is quite challenging. GABA is electrochemically inactive and has weak UV–visible absorption characteristics. Therefore, derivatization of this compound before analysis is required. Derivatizing agents commonly used are o-phthalaldehyde [4], 2-hydroxynaphthaldehyde [5,6], dabsyl chloride [7] and 9-fluorenylmethyl chloroformate [8]. However, these reagents have some drawbacks. For example, products obtained from derivatization with o-phthalaldehyde have poor stability at room temperature. Derivatization with dabsyl chloride and 2-hydroxynaphthaldehyde requires high temperatures. Moreover, HPLC analysis requires a long time, typically 15–40 min per chromatographic run. This technique requires expensive instrumentation and skilled technicians.

There are few chromophores that can react with GABA. Generally similar reagents used in HPLC are also exploited in spectrophotometric methods. Additionally, 7,7,8,8-tetracyanoquinodimethane has been proposed for detecting GABA [9] and an enzyme assay has been used for its measurement [10]. In the latter case, two enzymes were needed to catalyze conversion of GABA to succinic semialdehyde. Succinic semialdehyde was later reacted with NADP to produce NADPH which absorbs in the UV region. The main drawback of this method is that the resulting aldehyde must be separated prior to reaction with NADP. Thus, the method is tedious and time-consuming.

Recently, biosensors for detecting GABA have been proposed. Niwa et al. modified a glassy carbon electrode with bovine serum albumin-gabase-glutamate oxidase/osmium-poly(vinylpyrridine) that incorporated horseradish peroxidase [11]. Badalyan et al.

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immobilized GABA-aminotransferase (GABA-T) and aldehyde oxidoreductase in a polymer containing an osmium complex on a graphite electrode [12]. Zhou and Muthuswamy constructed an acoustic immunosensor by immobilizing a specific antibody on the gold surface of a quartz crystal electrode [13]. These biosensors had good detection limits for sensing GABA at micro-molar levels. However, electrode construction was somewhat complex. The biological substances are denatured easily under ambient conditions, which seriously hinder the stability of such electrodes.

Today, several nanometals have been used for colorimetric assays. Amongst these, silver nanoparticles (AgNPs) have gained much interest since they have a high extinction coefficient at a low cost. The reactions between particles and target analyte are generally based on electrostatic interactions [14–16], coordination chemistry [17], host-guest chemistry [18] or antibody-antigen binding [19]. These mechanisms cause the interparticle distance to decrease, leading to particle aggregation. As a result, the surface plasmon resonance (SPR) of the nanoparticles is shifted, and a change in the color of the colloidal solution is thus observed.

According to the literature [14–16], a positively charged compound can act as a 'molecular bridge' which initiates negatively charged nanoparticles causing them to aggregate via electrostatic attraction. In this work, GABA was prepared with a positive charge on its structure, so that it could induce negatively charged citrate capped AgNPs to aggregate. This resulted in a color change in the colloidal solution from yellow to green, which was spectrophotometrically monitored. This is the first report of the use of AgNPs for sensing GABA. The proposed method was used to detect GABA in pharmaceutical samples.

2. Experimental

2.1. Chemicals

All chemicals were of analytical grade. Deionized (DI) water (Zeneer Up 900, Korea) was used in all experiments. Silver nitrate, sodium borohydride, sodium citrate dihydrate and sodium acetate trihydrate were purchased from Sigma–Aldrich, USA. Gamma-aminobutyric acid (GABA) was acquired from Sigma–Aldrich, China. Glacial acetic acid (99.5% w/w) was obtained from Carlo Erba, UK.

A stock solution 2000 mg L^{-1} of GABA was prepared by dissolving 0.2 g of GABA in 100.0 mL of DI water. A series of working standard solutions was prepared by appropriately pipetting aliquots of the stock solution into 25 mL-volumetric flasks. A volume of 3.0 mL of acetate buffer was added to the individual flasks. Finally, DI water was added to fill the flasks to the 25 mL-mark.

To obtain 0.5 M acetate buffer at pH of 3.8, a 50 mL of 0.5 M sodium acetate trihydrate was mixed with 450 mL of 0.5 M acetic acid. The pH of solution was adjusted to 3.8 by addition of NaOH or HCl.

2.2. Preparation of AgNPs suspension

The AgNPs were prepared by reducing Ag^+ to Ag^0 with borohydride and stabilizing the nanoparticles with sodium citrate [20]. Briefly, 1.0 mL of 5 mM sodium citrate dihydrate was added to 39.0 mL of 0.64 mM $AgNO_3$ under vigorous stirring for 20 min. After that, 10 mL of 0.1% w/v $NaBH_4$ was added to the solution. A dark colloidal solution was obtained. This suspension was further stirred for an hour. The solution then became bright yellow. To facilitate calculation of nanoparticle concentration, the concentration of the as-prepared AgNPs was determined based on the final concentration of the $AgNO_3$ precursor solution. In this work, the AgNPs suspension was 0.5 mM. The suspension was allowed to stand for 2 h before use. The AgNP solution was stored under dark conditions at 4.0 ± 2.0 °C to maintain its stability for several weeks. For use in the colorimetric assay, the AgNP suspension was diluted to 0.2 mM prior to use.

2.3. Detection of GABA

A volume of 5.0 mL of the standard solution was thoroughly mixed with 5.0 mL of the 0.2 mM AgNP solution using a vortex mixer for 20 s. The UV–visible absorbance (V-630 Jasco, Japan) of the mixture was subsequently measured after 1 min.

2.4. Analysis of pharmaceutical samples

GABA dietary supplements were purchased from local drug stores. The GABA powder in one capsule was precisely weighted and then dissolved in 100.0 mL of water. This solution was filtered through a 0.22 μm nylon membrane. A 3.0 mL volume of filtrate was mixed with 3.0 mL of buffer before adjusting the total volume to 25.0 mL with DI water.

For GABA analysis, 5.0 mL of sample was added to 5.0 mL of a 0.2 mM AgNP solution. Absorbance was recorded after 1 min.

2.5. Validation method

To check the reliability of the method under study, the developed AgNP sensor was validated with a spectrophotometric method [6]. A 1.0 mL volume of GABA solution was transferred into 0.6 mL of borax buffer (pH 8), followed by addition of 2.0 mL of 0.3% w/v 2-hydroxynapthaldehyde. The solution was heated in a water bath at 80 °C for 10 min. Next, the solution was allowed to cool to room temperature before adjusting its final volume to 10.0 mL with methanol. Absorption spectra were measured at a wavelength of 415 nm.

3. Results and discussion

3.1. Principle for the GABA sensing

The synthesized AgNPs were characterized using transmission electron microscopy (TEM) and UV–visible spectrophotometry. The particles were spherically shaped with a diameter of 17 ± 4 nm. The colloidal solution appeared bright yellow. The maximum absorption was found at 390 nm and was attributed to the SPR of monodispersed AgNPs [20]. This was due to a negative electrostatic layer caused by citrate caps on AgNPs, forcing each particle to remain separate of neighboring particles. From the literature [14–16], a positively charged molecule can effectively trigger citrate capped nanoparticles to aggregate. Accordingly, GABA can also induce particle aggregation if it has a net positive charge. Under this assumption, positively charged GABA was prepared and subsequently interacted with AgNPs.

GABA, a molecule containing carboxylic and amino groups, has an isoelectric point (pI) of 7.30 [21]. Therefore, positive electrical charges can be generated when dissolving this molecule in a medium with pH lower than the pI value. In the current study, GABA was prepared in acetate buffer with a pH of 3.8. After introduction of the prepared GABA solution into the colloidal solution, the color of solution changed from yellow to green. The SPR absorption of AgNPs decreased and a new shoulder was observed at approximately 500–600 nm (Fig. 1). TEM images were used to verify the aggregation of AgNPs. For a comparison purposes, GABA in DI water was prepared. The pH of this solution was 6.8, which implied that GABA was primarily in the form of zwitterions. This solution was

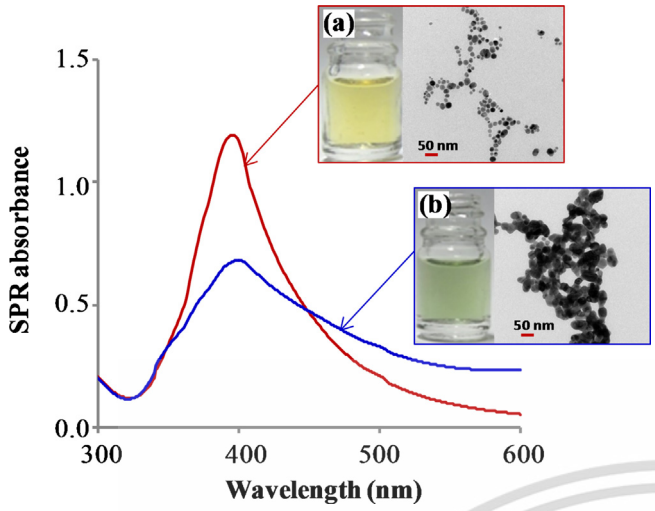


Fig. 1. SPR spectra, color and TEM images of AgNPs suspension in acetate buffer in (a) the absence and (b) presence of 300 mg L^{-1} of GABA.

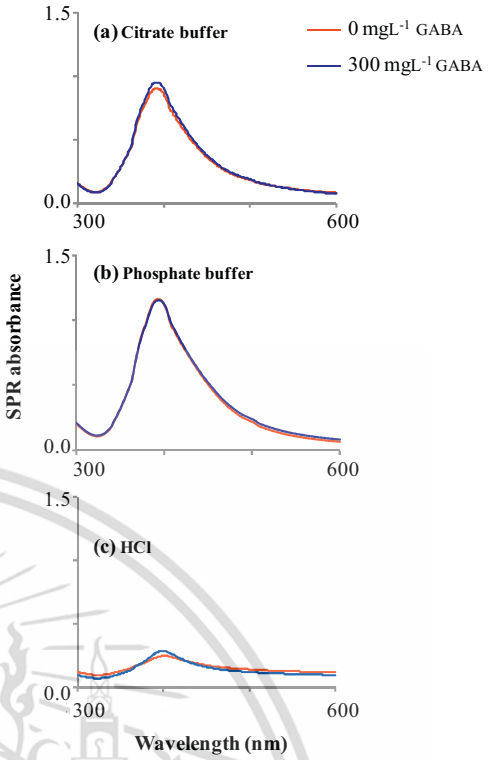
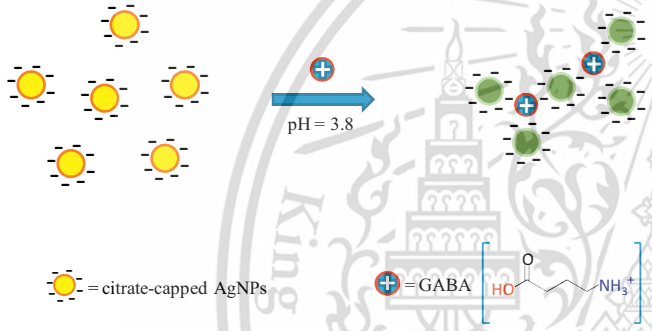


Fig. 2. SPR spectra of AgNP solutions in the absence and presence of 300 mg L^{-1} of GABA in media consisting of (a) citrate and (b) phosphate buffer, and (c) HCl.



Scheme 1. Schematic illustration of the colorimetric detection of GABA based on citrate-capped silver nanoparticles.

dispensed into the AgNP solution. As expected, the SPR intensity of AgNPs was not changed.

This result confirmed that the aggregation mechanism of AgNPs was caused by electrostatic interaction between negatively charged citrate stabilized particle surfaces and the positive charges of GABA. A schematic interaction is shown in Scheme 1.

3.2. Optimization of the experimental condition

AgNPs have their own unique SPR band, which strongly depends on size, shape, interparticle distance and the surrounding medium. In order to increase the detection sensitivity to this material, factors facilitating the SPR change in a presence of target analyte were critically studied.

Early in the study, a colloidal solution was prepared following [20] using 50 mM citrate as capping agent. It was found that the resulting particles were insensitive to GABA. This was probably due to excessive citrate causing a greater degree of particle dispersion. Consequently, GABA was electrostatically unable to induce these particles to form clusters. As shown in the Supporting information of Fig. S1, the SPR absorbance of AgNP suspensions exhibited small changes with and without GABA. The AgNP preparation was later modified to use 5 mM of citrate as a stabilizer. Under this condition, the resulting particles showed a superior capability to detect GABA (Fig. S1). The zeta potentials of AgNPs obtained by different preparation protocols were determined using a Zetasizer (Malvern Instruments, UK). The zeta potentials of particles stabilized by 5 and 50 mM of citrate were −0.118 and −0.260, respectively. This indicated that excessive amounts of citrate afforded a larger number of negative ions on particle surfaces which resulted in well-stabilized particles that were difficult to aggregate in the presence of GABA. In this work, 5 mM of citrate was used as a suitable capping agent.

The effect of the surrounding media on the reaction between GABA and AgNPs was investigated. The media under study included acetate, citrate and phosphate buffers, as well as hydrochloric acid. The SPR spectra of AgNP suspensions in different media before and after addition of GABA were compared. The SPR spectra of AgNPs suspended in acetate buffer declined after addition of GABA (Fig. 1). Whilst in citrate and phosphate buffers, the SPR spectra with and without GABA almost unchanged, indicating these media did not facilitate particle aggregation (Fig. 2a and b). Alternatively, the particles were readily aggregated in hydrochloric acid, even in the absence of GABA (Fig. 2c). Accordingly, acetate buffer was selected as a suitable medium.

The pH of media not only influenced the electrical charge of target analyte, but also particle stability [16,22]. In this work, various pH values less than the pI of GABA were examined. Standard solutions of GABA were prepared in acetate buffer at pH values of 3.8, 4.6 and 5.6. These solutions were then added to the AgNP solution. It was revealed that a medium with pH value of 3.8 provided a better response for detection of GABA than other conditions. Since citrate is a weak acid with three pK_a values at 3.1, 4.7 and 6.4, the solution pH affected the charge state of this ion. At higher pH values (4.6 and 5.6), carboxylates of citrate tended to be ionized, resulting in negatively charged particle surfaces. This promoted extensive repulsion between particles. The electrostatic interaction between positively charged GABA and negatively charged particles might have been insufficient to overcome the repulsion forces among particles. Therefore, aggregation of AgNPs was not attained. The SPR absorbance did not change (Supporting information of Fig. S2). However at pH 3.8, carboxylates are more protonated, decreasing the negative charges on particle surfaces. The interparticle distance

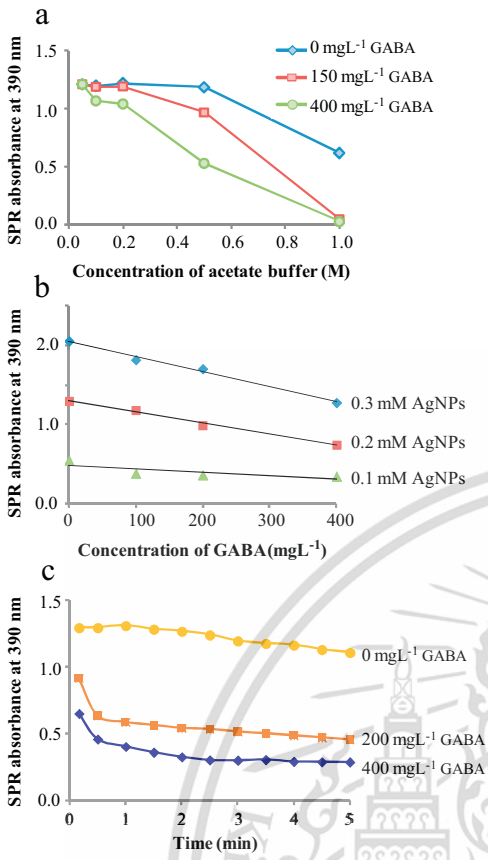


Fig. 3. Effects of (a) ionic strength, (b) concentration of AgNPs and (c) reaction time on the SPR absorbance of the corresponding reaction.

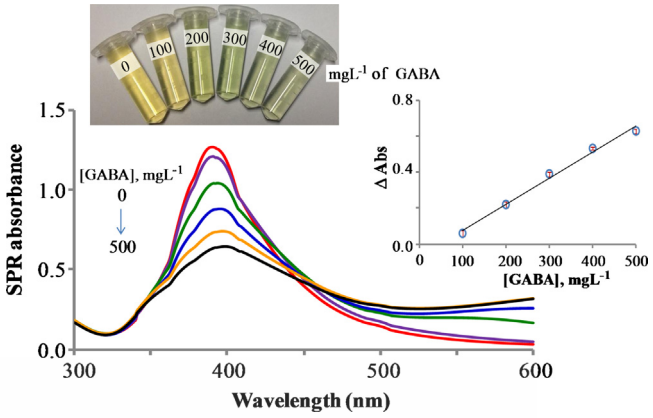


Fig. 4. SPR spectra and photographic image of AgNPs solutions with various concentrations of GABA. Inset is a linear relationship between the change of SPR absorbance (ΔAbs) and GABA concentration.

was shortened. When GABA was introduced into this suspension, particles rapidly aggregated. As a result, the SPR intensity gradually declined consistent with the concentration of GABA (Fig. S2). In order to confirm the above description, zeta potentials of particles at different pH values were investigated. It was found that the zeta potential of AgNPs dispersed in solution pHs of 3.8, 4.6 and 5.6 were −0.019, −0.067 and −0.083, respectively. These results reflected higher pH values and more ionized citrate ions obtained, leading to greater dispersion of particles with less sensitivity to GABA.

Ionic strength affects the aggregation process. Typically, charge repulsion of negatively charged nanoparticles can deteriorate in high ionic strength environments, due to the electrostatic shielding effect [23]. Consequently, self-aggregation of particles can occur even in absence of analyte. With this in mind, the concentration of acetate buffer was varied from 0.05 to 1.0 M. In Fig. 3a, when using 0.05 M buffer, the AgNPs did not aggregate although GABA concentrations of 150 and 400 mg L^{-1} were added. AgNP aggregation was more easily induced by GABA when the buffer concentration was increased to 0.5 M. This resulted in a decrease in SPR intensity at 390 nm after GABA addition. However, at buffer concentrations higher than 0.5 M, some agglomeration was found even though GABA had not yet been introduced. Hence 0.5 M acetate buffer (pH 3.8) was employed for the current study.

Further investigation focused on the concentration of AgNPs. In this work, the as-prepared AgNP concentration (calculated based on the final concentration of $AgNO_3$) was 0.5 mM. This suspension was diluted to 0.1–0.3 mM prior to reaction with GABA. The result showed that 0.1 mM of AgNPs gave poorer detection of GABA, while 0.2 and 0.3 mM promoted better sensitivity (Fig. 3b). To minimize reagent consumption, 0.2 mM of AgNP suspension was chosen for the assay.

The reaction time of the proposed method was determined by monitoring the decrease in the SPR absorbance as a function of time, after the colloidal solution was mixed with GABA. As shown in Fig. 3c, the absorbance decreased noticeably by 1 min. Then, absorbance gradually declined and remained constant after 3 min. Rapid of analysis is desirable. A detection time of 1 min was thus chosen.

3.3. Analytical performance of AgNPs sensing for GABA

Under optimal conditions, AgNPs were used as a colorimetric probe for quantitative determination of GABA. It can be seen in Fig. 4 that the color of AgNP solutions changed from yellow to green with increasing concentrations of GABA from 0–500 mg L^{-1}. Correspondingly, the SPR intensity of colloidal solutions at 390 nm decreased accompanied by broader peaks with a slight red shift. Simultaneously, a shoulder at approximately 500–600 nm emerged. A calibration curve was constructed by plotting the change in absorbance (ΔAbs) at the wavelength of maximum absorption versus the GABA concentration. The calibration was linear over the range of 100–500 mg L^{-1} (Fig. 4). A regression was done and found to be:

$$\Delta Abs = 0.0014\,[GABA, mg\,L^{-1}] - 0.068, R^2 = 0.990$$

The limit of detection (LOD) and quantification (LOQ) were determined from 3 and 10 times the standard deviation of the blank, respectively. Concentrations of 57.7 and 79.2 mg L^{-1} of GABA were found to be the LOD and LOQ of this method, respectively. Although the detection limit of the developed method was higher than that of the HPLC method [5,6], it is sensitive enough for determination of GABA levels in supplements. The reproducibility of the method was evaluated based on the relative standard deviation (RSD) of 300 mg L^{-1} of GABA (seven replicates). A RSD of 3.2% was obtained, indicating that the method provides good reproducibility of measurement.

3.4. Interference study

The influence of foreign species that are sometimes found in formulations of GABA supplements was studied. These included inorganic cations (Na^+, K^+ and Mg^{2+}) and organic compounds (glucose, fructose, glycine and vitamin B_6). At a concentration of 300 mg L^{-1}, all species tested, including GABA, were individually exposed to the AgNP sensor. As shown in Fig. 5, of the species

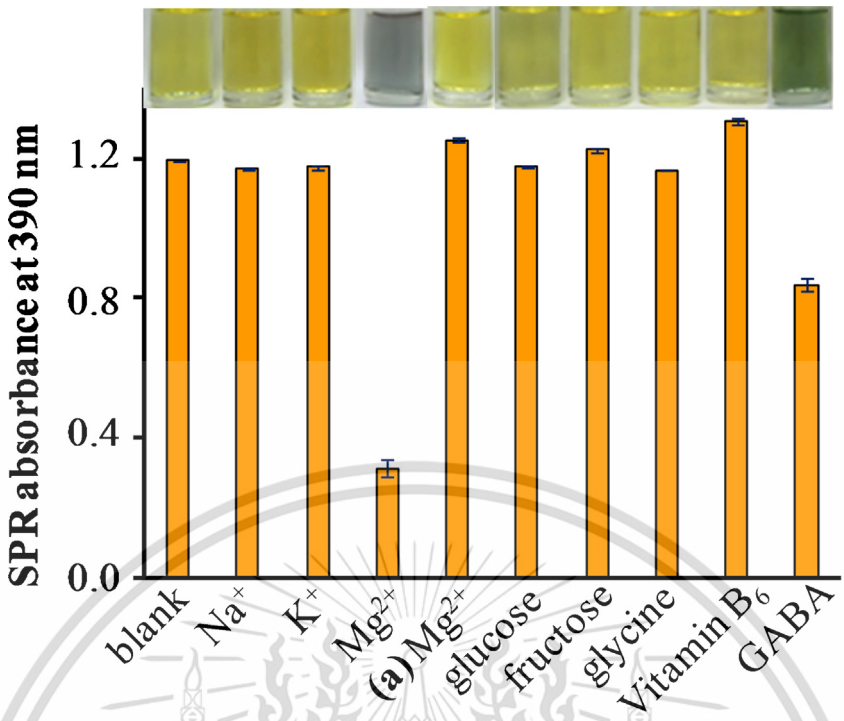


Fig. 5. SPR absorbance of AgNP sensor with the addition of 300 mg L^{-1} of ions/compounds. Only (a) Mg^{2+} is a concentration at 7.5 mg L^{-1}. The inset is an image of the corresponding color responses of the AgNP sensor to tested substances.

examined, only Mg^{2+} and GABA caused obvious changes in the SPR absorbance of AgNPs. This result indicated that Mg^{2+} could interfere with the assay. However, the amount of Mg^{2+} usually found in these supplements is very low (e.g., 62.5 mg Mg^{2+}/500 mg GABA capsule). With the sample preparation in Section 2.4, the final concentration of Mg^{2+} in the sample was determined to be 7.5 mg L^{-1}. Therefore, in a further study, 7.5 mg L^{-1} of Mg^{2+} was prepared and spiked into AgNP suspension. It was found that Mg^{2+} at this level could be tolerated and did not influence the determination of GABA. Hence, the AgNP sensor had good selectivity and could potentially be applied to determination of GABA in dietary supplements.

3.5. Analysis of pharmaceutical samples

To evaluate the feasibility of using the developed assay for determining GABA contents of dietary supplements, analytical recoveries were studied. Samples were prepared by spiking them with various concentrations of GABA. The results of this analysis are given in Table 1. The recoveries ranged from 97.5 to 104.5%, indicating that sample matrices did not interfere with this method.

For validation purposes, four supplement samples were analyzed using both the developed method and a spectrophotometric method employing 2-hydroxynaphthaldehyde derivatization [6]. These results are shown in Table 2. The results were statistically compared using a paired *t*-test. There was no significant difference between the results obtained from these two methods at a 95% confidence level ($t_{observed}$ = 0.57, $t_{critical}$ = 4.30). This shows that our new method for measuring GABA in dietary supplement is reliable. Compared to conventional HPLC methods [5,6] the developed method can be performed at room temperature without the use of a derivatizing agent. Moreover, the reaction proceeds quickly, requiring only 1 min per measurement. The method is inexpensive and more practical for routine use than an enzyme assay [10]. Unlike other sensors reported in the literature [11–13], this colorimetric AgNP probe is simple. It does not involve complicated electrode preparation and does not require high levels of technical skill.

Table 2
Comparison of the colorimetric AgNP sensor for quantification of GABA and a 2-hydroxynaphthaldehyde method.

Supplement	GABA content (mg per capsule)		
	Labeled	AgNP sensor	2-Hydroxynaphthaldehyde method [6]
A	750	742.8 ± 5.6	744.3 ± 7.2
B	750	727.2 ± 4.3	732.4 ± 1.6
C	500	498.8 ± 6.8	497.3 ± 5.1
D	500	504.5 ± 4.1	491.9 ± 2.1

4. Conclusions

This work presents a colorimetric method for detection of GABA based on aggregation of AgNPs. The detection principle relies on electrostatic interactions between positively charged GABA and

Table 1
Recovery results of GABA in dietary supplements using the developed AgNP sensor.

Sample	GABA concentration (mg L^{-1})			Recovery (%)
	Detected	Added	Found	
A	110.0 ± 4.6	100.0	210.7 ± 3.4	100.7
B	118.5 ± 2.2	200.0	327.5 ± 6.9	104.5
C	131.8 ± 3.4	100.0	236.3 ± 3.2	104.5
D	221.0 ± 6.4	100.0	318.5 ± 4.4	97.5

negatively charged AgNPs. This interaction induced particle aggregation results in SPR and color changes in the colloidal solution. Thus, the concentration of GABA could be determined using a spectrophotometer. The developed method has several remarkable advantages over conventional spectrophotometric [6,9,10] and chromatographic [4–8] techniques. The method is facile and low-cost. The reaction can be performed at room temperature and requires only 1 min. The results showed that the developed AgNP probe offers good precision and accuracy for quantification of GABA in pharmaceutical supplements. This method may be used for quality control in manufacturing products containing GABA.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2016.01.115.

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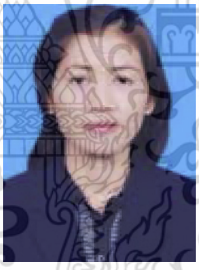
Biographies



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Original research article

Sequential injection for determination of gamma-aminobutyric acid based on its effect on second order light scattering of silver nanoparticles

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ABSTRACT

An automated sequential injection (SI) with second order light scattering (SOS) detection for determination of gamma-aminobutyric acid (GABA) was developed. Quantitation is based on electrostatic interaction between GABA and citrate-capped silver nanoparticles (AgNPs). In acetate buffer at pH 3.8, the positively charged GABA induces the nanoparticles to aggregate. This results in a change of light scattering monitored using a spectrofluorometer. In this work, working standard solutions of GABA were prepared in-line by the SI system pumping appropriate volumes of a stock solution of GABA and acetate buffer into a holding coil. Solution of AgNPs was subsequently drawn into the coil. The reaction zone was then transferred to the spectrofluorometer, set with excitation and detection wavelengths at 300 and 600 nm, respectively. Under optimised condition, the SOS intensity was proportional to the concentration of GABA. As a result, a linear curve was obtained in the range of 100–400 mg L^{-1} GABA, with a lower limit of detection of 39.6 mg L^{-1}. Good precision of analysis was achieved, with 0.6 and 3.3% relative standard deviation (RSD) for external calibration ($n = 5$) and standard addition ($n = 3$), respectively. The developed method was successfully applied for quantification of GABA in dietary supplements (2 samples) and samples of instant green tea (2 samples).

1. Introduction

Gamma-aminobutyric acid (GABA) is a non-protein amino acid found in certain foods, such as green tea and germinated brown rice, among others. GABA has been described as a 'health promoting substance'. It is claimed that it can diminish anxiety and promote relaxation (Abdou et al., 2006). There have been reports indicating that an intake of 80 mg GABA per day can significantly reduce blood pressure in humans (Matsubara et al., 2002). Continual use of GABA-enriched food is said to reduce sleeplessness (Okada et al., 2000). It was recently suggested that GABA may promote long-term memory and improved brain function (Thanapreedawat et al., 2013). In view of these purported health benefits, GABA has received interest by the food industry. Presently, GABA is fortified in some food products and purified GABA sold as a dietary supplement.

Various techniques have been used for measuring GABA concentration. These include liquid chromatography with pre-column derivatization (Khuhawar and Rajper, 2003), spectrophotometry (Li et al., 2010; Tanpramoon and Choengchan, 2014) and enzymatic assay (Zhang and Bown, 1997). These techniques are based on UV–vis detection. However, determination of GABA in food samples can be challenging. Many foods are coloured which may significantly interfere with UV–vis detection. Sample pre-treatment is therefore necessary to remove color pigments before

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analysis. Alternative methods for GABA detection have been proposed, such as electrochemical detection (Niwa et al., 1998) and acoustic impedance biosensor (Zhou and Muthuswamy, 2004).

Light scattering is a phenomenon that can be employed as an analytical technique. A common scattering mode is resonance Rayleigh scattering (RRS). In an RRS system, the wavelength of scattered light is the same as the wavelength of its excitation ($\lambda_{RRS} = \lambda_{Ex}$). In 1993, RRS was first utilised for detection of porphyrin on DNA (Pasternack et al., 1993) and was shown to be simple and sensitive. Later, an RRS-based method was developed for measurement of other species (Kong et al., 2014; Zhu et al., 2014). When a grating monochromator is employed there is also transmission of light at twice the wavelength of the selected wavelength. This is known as second-order diffraction or second-order light scattering (SOS), $\lambda_{SOS} = 2\lambda_{Ex}$ (Lakowicz, 2006; Sun, 2008). SOS was considered as interference in spectrofluorometric measurements of weakly fluorescing system. However in 1995, Liu's group demonstrated application of SOS for quantitative analysis (Liu et al., 1995a,b). It has been proposed as a novel analytical technique for trace analyses (Ding et al., 2005; Li et al., 2007).

Noble metal nanoparticles possess unique optical properties, such as surface plasmon resonance and strong light scattering. As a result, they are widely used in the fields of chemical and biomedical analysis as sensors. In recent years, silver nanoparticles (AgNPs) have been used as probes for determination of quinolones (Ding et al., 2006), ciprofloxacin (Zhao et al., 2008) and nitrate (Wang et al., 2009). These works employed measurement of second order scattering to measure analytes at the nanomolar level (Ding et al., 2006; Zhao et al., 2008).

Sequential injection (SI) (Ruzicka and Marshall, 1990) is one type of flow-based technique (Ruzicka and Hansen, 1988). Its essential feature is minimising of reagent consumption and waste production. The SI method is also superior in terms of robustness, reliability and high sample throughput. The method is amenable to computer control. Due to these advantages, the SI method has been used in a wide variety of applications, including environmental monitoring (Kaewwonglom and Jakmunee, 2015), quality control (Teerasong et al., 2010) and clinical diagnosis (Economou et al., 2007). Construction of calibration curves is necessary for quantitative analysis. Construction of the curve involves laborious preparation of a series of standard solutions. SI can use in-line preparation of standard solutions from a single stock standard solution (Marcelo et al., 2002). The approach is based on aspiration of variable volumes of the standard solution and solvent into a holding coil of the system, under a programmable computer controller. In-line preparation of solutions by a SI system can be used for either external calibration or standard addition method (Fernandes et al., 2001; Marcelo et al., 2002), as well as for sample dilution (Themelis et al., 2004).

This work aimed to develop a sequential injection incorporated with second order scattering (SOS) detection for determination of GABA concentrations. The detection relies on the electrostatic attraction between positively charged GABA and negatively charged citrate-capped silver nanoparticles in acetate buffer in which the nanoparticles are readily aggregated. When the particle size increases, SOS intensity is enhanced. The concentration of GABA can be quantified by monitoring the relative change in SOS intensity using a spectrofluorometer. Unlike UV–vis detection, sample colour poses no difficulty in analyses using SOS detection. The proposed method was applied to quantitation of GABA in a food supplement and a green tea powder.

2. Materials and methods

2.1. Preparation of GABA solution

All solutions used in this work were prepared from analytical grade chemicals and deionized (DI) water (Zeneer Up 900, Human Corporation, Seoul, Korea).

A 0.5 M acetate buffer at pH of 3.8 was prepared from acetic acid (Carlo Erba, Rodano, MI) and sodium acetate trihydrate (Sigma-Aldrich, St. Louis, MO). A 50 mL volume of 0.5 M sodium acetate and 450 mL of 0.5 M acetic acid were mixed. The mixture was adjusted to pH 3.8 by addition of 1.0 M NaOH.

A stock solution of 500 mg L^{-1} of GABA was obtained by weighing 0.05 g of gamma-aminobutyric acid (≥99%) (Sigma-Aldrich) and dissolving in 100.0 mL of 0.5 M acetate buffer.

2.2. Synthesis of AgNP colloidal solution

The AgNP solution was prepared following a published method (Qu et al., 2012) with minor modification. A 0.64 mM $AgNO_3$ solution (Carlo Erba) was used as the precursor. 1% w/v $NaBH_4$ and 5 mM sodium citrate (both Sigma-Aldrich) were used as reducing and capping agents, respectively. A 1.0-mL aliquot of sodium citrate was added to 39.0 mL of $AgNO_3$ precursor. This solution was vigorously stirred for 20 min. Then 10.0 mL of $NaBH_4$ was added

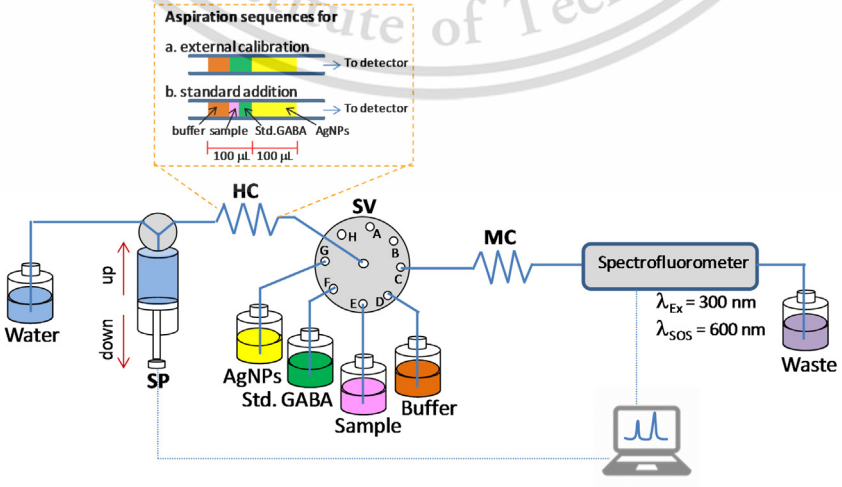

Fig. 1. A configuration of sequential injection with second order light scattering for determination of GABA. SP: syringe pump, HC: holding coil (1 mm i.d. × 50 cm length), SV: eight-port selection valve, MC: mixing coil (1 mm i.d. × 50 cm length) and D: spectrofluorometer (λ_{Ex} = 300 nm, λ_{SOS} = 600 nm).

dropwise to the solution. Stirring was continued for an hour, after which it was allowed to stand for 2 h before use. The particles remained well dispersed in suspension for a month when stored in the dark at ~4 °C.

The concentration of the AgNP suspension, calculated based on the final concentration of $AgNO_3$ precursor, was 0.5 mM. This suspension was diluted to 0.4 mM prior to use in the SI method.

2.3. Sample preparation

The powder in one capsule (~0.75 g) of GABA supplement was weighed. Then 100.0 mL of DI water was added to dissolve the powder. The solution was filtered through Whatman No.1 filter paper and then a 0.22 μm nylon membrane. Appropriate dilution (~50 times) was made by addition of buffer to obtain a GABA concentration within the calibration range. This sample was clear and colourless.

Green tea powder (3 g) was weighed and dissolved in 50.0 mL of hot DI water (~70 °C). The solution was centrifuged at 6000 rpm for 20 min. The supernatant was filtered through filter paper and a 0.22 μm nylon membrane. Buffer was used to dilute the sample (~1.5 times) before analysis. The green tea sample was pale yellow-green in appearance.

In this work, 2 dietary supplements and 2 samples of instant green tea were analyzed. Supplements were purchased from local drug stores. Green tea samples were products of Japan, which purchased from the Internet.

2.4. Assembly of the SI system

Fig. 1 illustrates the SI configuration. It comprises a syringe pump (SP) (Hamilton Company, Reno, NV) equipped with a 5-mL syringe and an eight-port selection valve (SV) (Hamilton Company). A holding coil (HC) made of polytetrafluoroethylene (PTFE) tubing (1.0 mm i.d. × 50 cm) is connected to the pump and the centre port of the SV. Another 50 cm of the same size tubing is used as a mixing coil (MC). A Jasco FP-8200 spectrofluorometer (Jasco Corporation, Tokyo, Japan) with a Hellma 176.052QS flow-through cell (Hellma Analytics, Müllheim, Germany) is employed to detect the SOS. The excitation wavelength (λ_{Ex}) was set at 300 nm and the SOS intensity was recorded at 600 nm (λ_{SOS}).

2.5. Operation of the SI system

For determination of the GABA concentration in the supplement, an external calibration method was used. Working standard solutions were prepared in-line by loading different volumes of 500 mg L^{-1} GABA, viz., 0, 20, 30, 40, 60 and 80 μL, into the HC (via port F of SV, see Fig. 1). Buffer solution was pumped into the HC coil (via port D) to adjust the final volume to 100 μL. The final GABA concentrations were 0, 100, 150, 200, 300 and 400 mg L^{-1}, respectively.

The green tea sample contained several compounds that can interfere with the analysis. To eliminate this problem, standard addition method was used. A 10 μL volume of the green tea sample was drawn into the HC (via port E). Various volumes of the stock GABA solution and buffer were aspirated into the coil to bring the total volume in the coil to 100 μL.

In this work a 100 μL volume of AgNP solution was used for the reaction. The flow rate for transferring solution (both aspiration and dispensing) was fixed at 20 μL s^{-1}. Table 1 shows the operation steps of the SI system for the standard addition method. The external calibration method employs the same steps as in Table 1, but omitting step 3 of the operation.

2.6. Comparison of methods

An HPLC method (Khuhawar and Rajper, 2003) was employed for comparison purpose. A 1.0 mL aliquot of GABA solution was added into 0.6 mL borax buffer (pH ~8) followed by addition of 1.0 mL of 2-hydroxynapthaldehyde (0.3% w/v in methanol). The mixture was heated on a water bath at 80 °C for 10 min. This solution was allowed to cool then adjusted to a final volume of 5 mL with methanol. Then, 5 μL of solution was injected into the HPLC system. HiQ sil™ C18 column (4.6 mm i.d. × 150 mm; KYA Technologies Corporation, Tokyo, Japan) was used as stationary phase and a mixture of methanol:water (62:38 v/v) was used as mobile phase. Flow rate of the system, controlled by Waters 515 pump (Waters Corporation, Milford, MA), was fixed at 1 mL min^{-1}. Column temperature was set at 25 °C. The UV detector was set at 330 nm. In the HPLC method, a calibration curve in the range of 25–125 mg L^{-1} GABA was constructed.

The results obtained by the SI method were compared with results from the HPLC method using a paired *t*-test.

3. Results and discussion

3.1. Characteristics of AgNPs

The as-prepared AgNP suspension was yellow with maximum absorption at 390 nm, consistent with literature reports (Qu et al., 2012). Using transmission electron microscope (TEM) (Tecnai G^2 20; FEI Company, Hillsboro, OR), the particles were found to be spherical with a mean diameter of 17 ± 4 nm.

3.2. Interaction of GABA and AgNPs

Positively charged compounds can induce negatively charged stabilised nanoparticles to agglomerate. This interaction has therefore been applied for detection of many positively charged species (Gao et al., 2014; Gobel et al., 2016; Ling et al., 2008). In the current study, GABA was prepared with positive charge by adjusting the pH of the solution. GABA possesses an isoelectric point of 7.30 (Greenstein and Winitz, 1961). If the pH of solution is

Table 1
Operational sequences of sequential injection for determination of GABA by standard addition method with in-line standard solution preparation.

Step	Direction of piston of SP	SV position	Action	Volume (μL)
1	down	–	aspirate deionised water into syringe pump	2200
2	down	D	aspirate buffer solution into holding coil	varied[b]
3	down	E	aspirate sample containing GABA into holding coil	10
4	down	F	aspirate 500 $mgL^{-1}$of GABA standard solution into holding coil	varied[a]
5	down	G	aspirate AgNPs suspension into holding coil	100
6	up	C	dispense the mixture in holding coil towards detector	2400

[a] The volumes of the GABA stock solution were aspirated into the holding coil as 0, 20, 30, 40, 60 and 80 μL, respectively.
[b] The volume of buffer was varied to adjust the total volume of GABA solution in the holding coil to 100 μL.

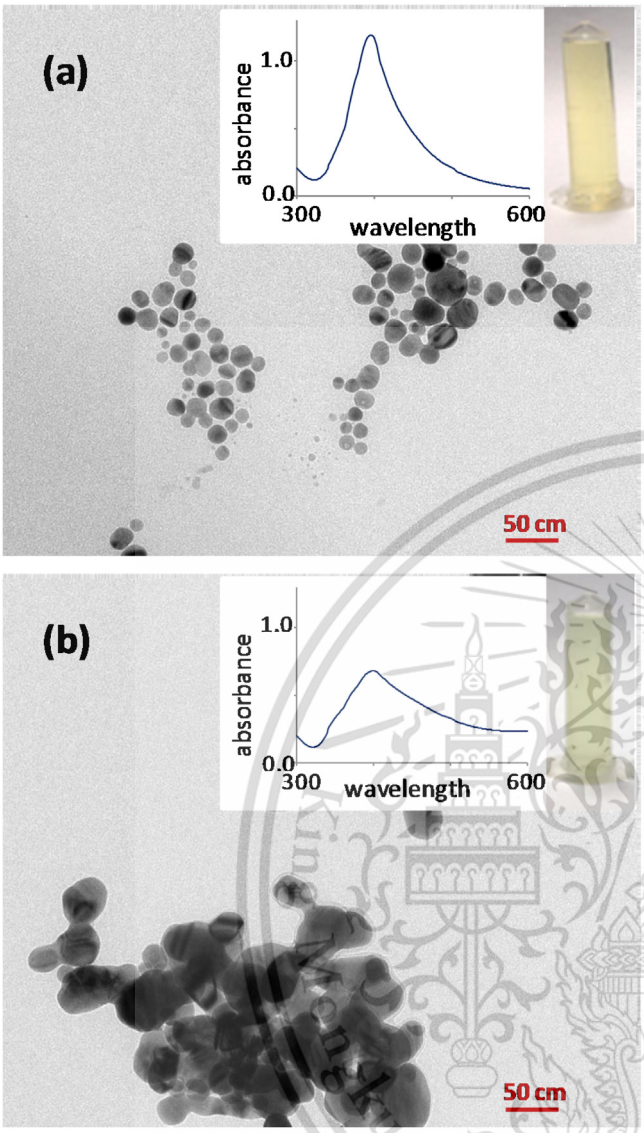

Fig. 2. TEM images of silver nanoparticles with (a) absence and (b) presence of 300 mg L^{-1} GABA in acetate buffer. Insets show the corresponding absorption spectra and color of the suspension. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lower than 7.30, GABA will be cationic. In this work, GABA was prepared in 0.5 M acetate buffer with a pH of 3.8. The aggregating reaction was observed when the pH-controlled GABA solution was pipetted into the AgNP suspension. The colour of the colloidal solution changed from yellow to blue-green (Fig. 2). The absorption band at 390 nm also noticeably decreased.

3.3. SOS characteristics

The SOS spectra of GABA, AgNPs and a mixture of GABA and AgNPs solutions were measured using a batch experiment. Excitation was set at 300 nm and the emission spectra of the solutions recorded over the wavelength 450–750 nm. The results (Fig. 3) showed that the SOS intensity at 600 nm of the GABA solution was very weak. The AgNP suspension showed a more intense SOS scattering. The SOS intensity improved significantly after addition of GABA. Based on this finding, it is possible to use

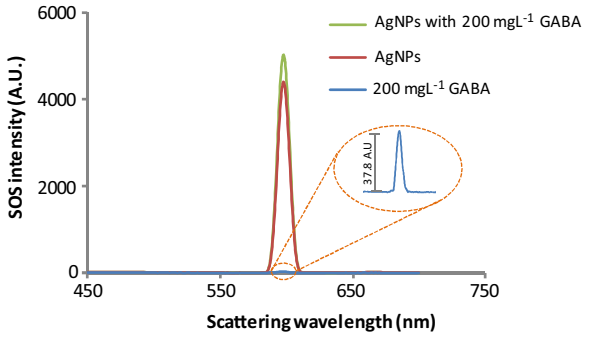

Fig. 3. Emission spectra of solutions of GABA, AgNPs, and mixture of GABA and AgNPs, with excitation wavelength at 300 nm.

the AgNPs as a probe for detection of GABA based on SOS measurements.

3.4. Optimisation of the SI method

A suitable wavelength for excitation was first examined. The excitation wavelength was varied from 225 to 350 nm. Intensity of the second order scattering (SOS) was measured at the corresponding double wavelength of excitation, i.e. $\lambda_{SOS} = 2\lambda_{Ex}$. It was found that the maximum SOS intensity was achieved when the excitation wavelength was 300 nm (Fig. 4a). Thus, 300 and 600 nm

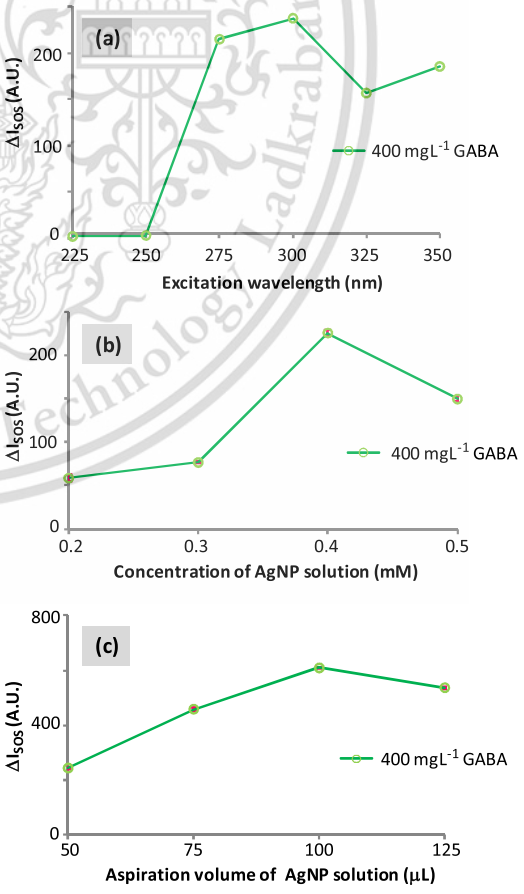

Fig. 4. Effects of (a) excitation wavelength, with emission intensity at $\lambda_{SOS} = 2\lambda_{Ex}$. (b) concentration and (c) volume of AgNP suspension on the SOS intensity (λ_{SOS} = 600 nm). ΔI_{SOS} is the difference in the SOS intensity with and without GABA.

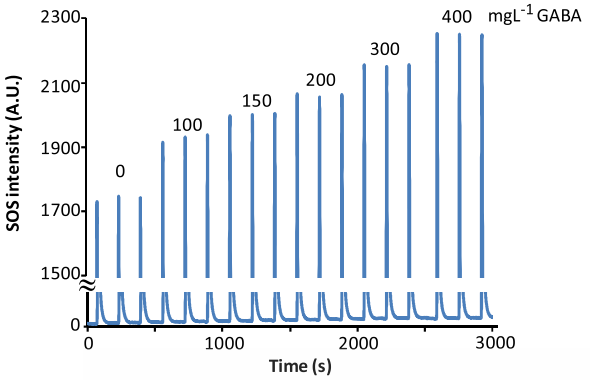

Fig. 5. Examples of signal profiles obtained by the sequential injection in Fig. 1 for external calibration.

were employed as the excitation and SOS detection wavelengths, respectively.

The next parameter investigated was the aspiration sequence. The following sequences were tested: (i) buffer-GABA-AgNPs, (ii) GABA-buffer-AgNPs and (iii) GABA-AgNPs-buffer. Sequence (i) gave the greatest reproducibility of the SOS signal. Hence, buffer-GABA-AgNPs was the aspiration sequence chosen for the final protocol.

The flow rate for dispensing the solution through the detector was studied. Flow rates of 10 and 20 $\mu L\,s^{-1}$ were compared. Detection signals using these two flow rates were not significantly different. Flow rates above 20 $\mu L\,s^{-1}$ were also tested. However, air bubbles sometimes were observed in the system at these higher flow rates and caused irreproducible signals. The flow rate of 20 $\mu L\,s^{-1}$ was selected since it leads to higher sample throughput.

Different lengths of mixing coil were examined, i.e., 50, 100 and 150 cm. Shorter mixing coils provided larger signal. Longer mixing coil may lead to more longitudinal dispersion of reaction zone thereby lowering the sensitivity. For this reason, the 50-cm length of coil was used for the final system.

Buffer concentration is a parameter that greatly affects the stability of nanoparticles. Therefore, the effect of the concentration of acetate buffer was investigated over the concentration range of 0.01–1.0 M. It was found that 0.01–0.1 M buffers were insufficient to induce AgNP aggregation even in the presence of GABA. Alternatively, when using 1.0 M buffer, clusters of particle readily formed without addition of GABA. In a high concentration of buffer, the particle surfaces are surrounded with large numbers of ionic species. These ions can decrease the electrostatic repulsion among particles, causing aggregation even without the target analyte (Trieu et al., 2014). In this work, the optimal buffer concentration for supporting AgNP aggregation in the presence of GABA was found to be 0.5 M. At this buffer concentration, the SOS intensity increased proportionally with the concentration of GABA.

Concentration and volume of the AgNP reagent could affect SOS intensity. Effects of these parameters were thus studied. Concentrations of AgNPs were varied from 0.2 to 0.5 mM. The SOS signal was progressively enhanced when AgNP concentration was increased from 0.2 to 0.4 mM (Fig. 4b). The degree of particle agglomeration is generally related to the number of particles in a unit volume (Ding et al., 2006; Guo et al., 2007; Zhao et al., 2008). With larger numbers of particles, agglomeration was easier to initiate. However, it was surprisingly observed that the SOS signal declined when AgNP concentration was increased to 0.5 mM. With a large excess of AgNPs, the ratio of analyte to particles is small. This makes it difficult to initiate particle agglomeration (Guo et al., 2007). Therefore, 0.4 mM AgNP solution was used for the final method. The volume of AgNP reagent loaded into the system was varied from 50 to 125 μL. In Fig. 4c, an AgNP solution of 100 μL gave the highest SOS intensity. Consequently this volume was adopted for the method.

3.5. System performance

Using the system in Fig. 1 under optimised conditions, well-defined signal profiles were achieved (Fig. 5). External calibration was performed and a calibration curve was constructed, plotting the change in SOS intensity (ΔI_{SOS}) against GABA concentration. A linear relationship was found for the concentration range of 100-400 mg L^{-1} GABA. The regression equation was $\Delta I_{SOS} = (0.931 \pm 0.035)[\text{GABA, mg L}^{-1}] + (93.7 \pm 9.08)$, with regression coefficient (r^2) = 0.995. The limit of detection (LOD) and limit of quantitation (LOQ) were defined as 3 and 10 times the standard deviation of signal generated by 200 mg L^{-1} GABA (5 replicates). The LOD and LOQ were 39.6 and 132.0 mg L^{-1} GABA, respectively. Reproducibility of the method was evaluated from the relative standard deviation (RSD) (Skoog et al., 2004) at 200 mg L^{-1} GABA signal (5 replicates). A%RSD of 0.6% was obtained, indicating that the method provided good reproducibility of measurement. The throughput of analysis was found to be 25 h^{-1} for external calibration.

Standard addition method was carried out using the same concentration range of standard solution as for the external calibration. The r^2 of individual standard addition lines was >0.99. Acceptable precision of analysis was achieved (RSD = 3.3%, 3 replicates). A comparison of GABA detection methods is shown in Table 2.

3.6. Evaluation of GABA contents in samples

The developed method was applied to the determination of GABA in dietary supplements and instant green tea samples. In the supplements, GABA is a major component of the formulation and thus categorized as a low matrix sample. External calibration was employed for analysis. Green tea sample contains many ingredients and thus standard addition method was employed. The

Table 2
Comparison of GABA detection methods.

Technique	Detection	LOD	%RSD	Sample	Reference
HPLC	UV–vis absorption	0.56 mg L^{-1}	2.4	cerebral spinal fluid	Khuhawa and Rajper, 2003
HPLC	UV–vis absorption	–	1.57	rice wine	Liu et al., 2015c
spectrophotometry	UV–vis absorption	0.27 mg L^{-1}	<2	dietary supplement	Tanpramoon and Choengchan, 2014
biosensor	cyclic voltammetry	0.01 mg L^{-1}	–	cultured nerve cells	Niwa et al., 1998
biosensor	acoustic impedance	3.92 mg L^{-1}	–	–	Zhou and Muthuswamy, 2004
this work	scattering	39.6 mg L^{-1}	0.6, 3.3	dietary supplement, green tea	–

LOD = limit of detection.
RSD = relative standard deviation.

Table 3
Comparison of GABA contents determined by the developed sequential injection (SI) and HPLC methods.

Sample	Analytical approach	GABA (mg) per capsule or sachet		
		Labelled	SI method ($n = 3$)	HPLC method ($n = 3$)
Dietary supplement 1	External calibration	750	771.3 ± 7.1	781.5 ± 10.2
Dietary supplement 2	External calibration	750	753.4 ± 4.5	736.4 ± 2.5
Instant green tea 1	Standard addition	20	19.6 ± 6.9	21.1 ± 1.9
Instant green tea 2	Standard addition	20	25.1 ± 2.3	21.0 ± 2.4

n = number of replicates.

analytical results are summarised in Table 3. Good agreement between two sets of results was attained at a 95% confidence level ($t_{observed} = 0.96$, $t_{critical} = 4.30$). Recoveries were measured by spiking 100 mg L^{-1} GABA into samples prior to analysis. Satisfactory recoveries were obtained in the range of 93.1–97.5% for the supplements and 99.4–102.2% for green teas samples.

The advantage of this method is that it can be applied for quantification of GABA in coloured food samples. This SI method is rapid, automated and reliable. Moreover, the analysis consumed low volumes of sample and AgNP reagent, with reduced waste production.

4. Conclusions

A new sequential injection (SI) method with second order light scattering (SOS) was developed for evaluation of GABA in food products. The SI system allowed automated preparation of working standard solutions, which significantly reduced manual labour. In the reaction, GABA induced AgNPs to aggregate, causing an increase in the intensity of second order light scattering. The SOS intensity increased proportionally with the concentration of GABA. Use of light scattering detection has the potential to analyse GABA directly in coloured samples. The developed method is reproducible, rapid and sensitive. Therefore, it may be an alternative method for quality control in the manufacture of GABA in foods and beverages.

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Publication:

1. Jinnarak, A. and Teerasong, S., A novel colorimetric method for detection of gamma-aminobutyric acid based on silver nanoparticles, Sensors and Actuators B: Chemical, 229 (2016) 315–320.
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