Green analytical method for determination of iron and ascorbic acid using crude extracts from betel nut (*Areca catechu* Linn.) as a natural reagent

Bordin Weerasuk

A Thesis Submitted in Partial Fulfillment of Requirements for
degree of Master of Science in Chemistry
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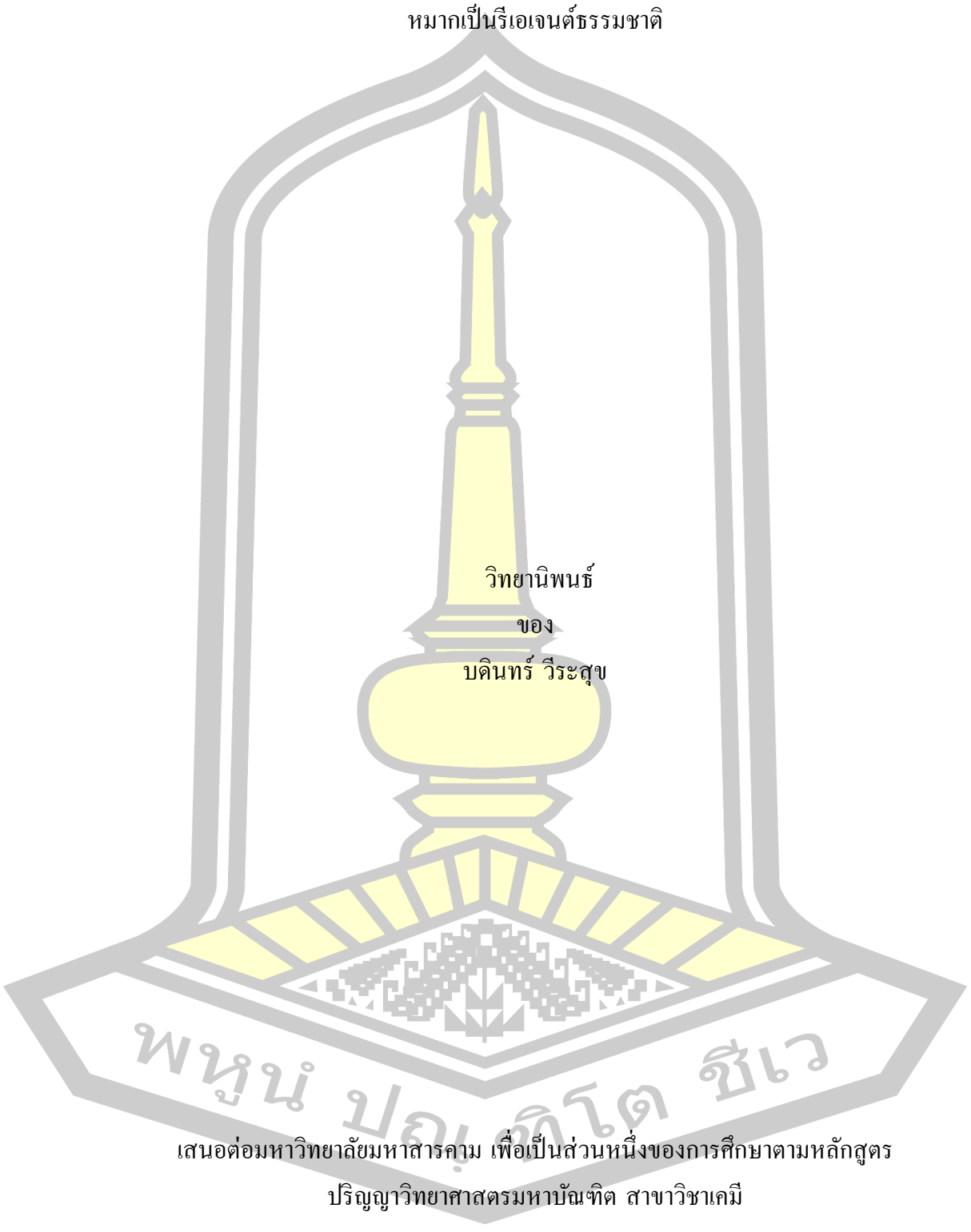

วิทยานิพนธ์

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TITLE Green analytical method for determination of iron and ascorbic acid using crude extracts from betel nut (*Areca catechu* Linn.) as a natural reagent

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ABSTRACT

In this work, an alternative less-harmful reagent from crude extracts of betel nut (*Areca catechu* Linn.) was proposed for determination of iron(III) and ascorbic acid by sequential injection spectrophotometric method. This method for iron(III) analysis is based on the reaction of iron(III) and betel nut extracted reagent in acetate buffer at pH 5.5 to provide an iron(III)- betel nut complex, which showed a maximum absorption at 565 nm. Determination of ascorbic acid is based on the reduction reaction of iron(III) to iron(II) by ascorbic acid resulting to decrease of absorbance of iron(III)-betel nut complex. Chemical and physical parameters such as pH, buffer concentration, volume of reagents, sample volume, mixing coil length and flow rate were investigated and optimized. Under the optimum conditions, a linear calibration graph in the range of 0.2–10.0 mg L^{-1} iron (III) was obtained with a correlation coefficient (r^2) of 0.9995. Limit of detection (LOD) and limit of quantification (LOQ) were 0.06 and 0.20 mg L^{-1}, respectively. The relative standard deviation (%RSD) of the method was less than 5.0% (n = 10, 6 days). The percentage recovery was found in the range of 81.20-103.75% for determination of iron(III). The linear calibration over the range of 4-50 mg L^{-1} ascorbic acid was obtained with a correlation coefficient (r^2) of 0.9981. Limit of detection (LOD) and limit of quantification (LOQ) were 1.20 and 4.00 mg L^{-1}, respectively. The relative standard deviation of less than 5.0 % (n=10, 5 day) and percentage label amount of 95.91-100.01 % for determination of ascorbic acid. The proposed method was successfully applied for determination of iron content in rice, vegetable and water samples with sampling rate of 40 h^{-1}. The results agreed well with those obtained from FAAS technique at the 95% confidence level. Moreover, this developed method was utilized to determine ascorbic acid content in pharmaceutical samples with sampling rate of 17 h^{-1}. The results agreed well with those obtained for titration method at the 95% confidence level.

Keyword : Iron(III), Ascorbic acid, Betel nut, Areca catechu Linn., Natural reagent, Green chemistry, Sequential injection spectrophotometry

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CHAPTER I

INTRODUCTION

1.1 Problems and provenance

Iron is an element in the transition metal group. In nature found two different forms such as ferrous; iron(II) and ferric; iron(III). Iron is a component of muscle and blood. It is essential to carry oxygen about the body (Jaikrajang et al. 2018). Iron is general found in water and food as liver, beef, pork, soybean etc. and also found in pharmaceutical form such as ferrous fumarate (Pragourpun et al. 2018). If the body among iron in small amounts, it may result in anemia hyperactivity, neurological disorders but if the body receive high level of iron, it may result in irregularity of the organs such as cancer, heart disease and other diseases as endocrine problems, arthritis, diabetes, and liver disease (Elci et al. 2008). Therefore, the body should receive the proper level of iron for safety and good health.

Recently, iron can be found in rice and vegetables, which are contaminated and absorbed from soils and water. Rice is the primary staple food for more than half of the world population. Therefore, the development of rice varieties with good nutritional quality will benefit more consumers. This new rice variety is considered as a source of high iron, which, that is sinlek rice. Sinlek rice still has many health benefits, which has been reported to control blood sugar levels and reducing total cholesterol levels and the risk of coronary heart disease in patients with type two diabetes (Chaiyakul et al. 2016). When considering the health benefits, sinlek rice is an alternative way to obtain the health of food. There is also hom-nin rice and brown rice as an alternative for good health. However, iron is potentially toxic in an excessive concentration (the dose of 0.8 mg kg^{-1} of body weight) was established as provisional maximum tolerable daily intake (FAO/WHO Expert Committee on Food Additives, 1983). Consequently therefore, the determination of iron in rice is an important for public health. Vegetables constitute essential diet components by iron, calcium and other nutrients. Metals in vegetables can be a direct threat to human health. A security limit for iron set by the World Health Organization (World Health Organization, 2003) for vegetable is 15 mg kg^{-1}.

Moreover, water forms the basis of all life on the earth is the foremost primary basic foodstuff for all kinds of civilization. There are many standards for the quality of drinking water. In general, iron in water comes from the dissolution of iron compounds in soil, leaching into groundwater that is distributed to wells and aquifers used in drinking water. The presence of iron in water effect characteristics such as

bitter and metallic taste and high concentration a precipitation. A security limit for iron set by the World Health Organization for drinking water and groundwater is 0.3 mg L^{-1} and ranges typically between 0.5 and 10.0 mg L^{-1}, respectively (World Health Organization, 2003). It is well known that water is beneficial for agricultural purposes.

The analytical techniques commonly used for the determination of iron are inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Sreenivasa et al. 2002), stripping voltammetry (Ghoneim et al. 2010), inductively couple plasma mass spectrometry (ICP-MS) (Aydin et al. 2010), flame atomic absorption spectrometry (FASS) (Amorim et al. 2016), graphite furnace atomic absorption spectroscopy (GFAAS) (Adolfo et al. 2019), inductively coupled plasma optical emission spectrometry (ICP-OES) (Gamela et al. 2019). Although these methods are of achieving highly sensitive detection, identification and quantification of heavy metals but they always require the supporting by laboratories and skilled operators (Jaikrajang et al. 2018). Moreover, they are very expensive. Therefore, alternative inexpensive method for determination of iron using spectrophotometric method are widely used for determination of iron due to simplicity, rapidity, low cost and wide applications with various complexing agents such as 1,2-dihydroxy-3,4-diketocyclo-butene (squaric acid) (Stalikas et al. 2003), 1,10-phenanthroline (Tesfaldet et al. 2004), 2-(5-bromo-2-pyridylazo)-5-[N-n-propyl-N-(3-sulfopropyl)amino]aniline (5-Br-PSAA) (Ohno et al. 2006), 2,6 bis(1-hydroxy-2-naphthylazo) pyridine (PBN) (Sharma et al. 2009, 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol(5-Br-PADAP) (Filik et al. 2012), 8–hydoxyquinolone (Adebayo et al.2011) and thiocyanate (Verma et al. 2017). Utilizing commercial synthetic reagents have many disadvantages such as hazardous and/or expensive, and generate hazardous chemical waste that, harmful to human health and potentially cause further environmental problems. Therefore, developing a novel method that is cheap, fast and uses a small amount of chemicals have been of interested. It is well known that common active ingredients found in plant extracts include phenolic compounds as flavonoid, tannin, lignin and others. Phenolic compound contain hydroxyl group (-OH) bonded directly aromatic ring. Found that, it responds for binding to or chelating with metal ions (Çakar et al. 2016) (Jaikrajang et al. 2018). Therefore, plant that contain phenolic compound may be used as sources of metal ion complexing agents.

Recently, the development in green analytical techniques and environmentally friendly for determination of iron using flow-based analysis techniques are the ways to achieve green analysis. SIA was developed by J. Ruzicka and G. Marshall in 2000 as an alternative to FIA. It has become an attractive analytical technique that has been widely used with spectrophotometric detection due to its general availability in laboratories, low cost, low reagent consumption, low waste production, cost-

effectiveness, small sample volume, and automation with numerous and widespread applications in quantitative analysis.

The use of natural reagents in conjunction with a flow-based system can confer a number of advantages. Using of natural reagents from plants extraction has been reported such asguava leaves. (Settheeworrarit et al. 2005), green tea (Pinyou et al. 2010), ma-kham-pom (Jaikrajang et al. 2018) extracts for analysis of iron, indian mulberry root (Tontrong et al. 2010), indian almond leaves (Insain et al. 2013) and heartwood of sappan wood (Siriangkhawut et al. 2016) extract for analysis of aluminium, pea extract for analysis of mercury (Gao et al. 2006), smilax china extract for analysis of iron and manganese (Ganranoo et al. 2019). However, there are not reported as green analytical method approaches in the literature using of betel nut extract as an alternative regent. Betel nut is one of the popular traditional herbal medicine uses in Thailand. The activities of it are anthelmintic, antifungal, antibacterial, antioxidant and anti-inflammatory (Rathod et al. 2015). Tannin are rich in the seeds (Wang et al. 1997), it compose many hydroxyl groups especially well-known for its complex ability with iron(III) ion. Therefore, betel nut extracts may be used as a complexing agent of iron(III). Utilizing of natural reagent of analysis has many advantages such as less-harmful reagent, important aspect in sustainable analytical chemistry, and the minimization of waste products.

Ascorbic acid is also known as vitamin C. It is a group of essential water-soluble (Kukoc-Modun et al. 2012) vitamins that can be found in biological and food systems such as fresh fruits and vegetables (Porto et al. 2019). Ascorbic acid is important in synthesis collagen biosynthesis, iron absorption and stimulates the immune system response and contributes to wound healing and osteogenesis. It also acts as an effective antioxidant that fights disease caused by free radicals. However, an ascorbic acid excess can lead to gastricirritation, and the metabolic product of vitamin C can cause renal problems. Ascorbic acid is a reducing agent of iron(III) to iron(II) (Bazel et al. 2018). The analytical techniques have been proposed for its determination of ascorbic acid such as chemiluminescen (Zhang, Qin 1996), voltammetry (Danet et al. 2008), spectrophotometry (Bazel et al. 2018 ; Elmagirbi et al. 2012) and chromatography (Zuo et al.2015; Ross 1994; Silva 2005).

Therefore, this work the development of an automation sequential injection (SI) spectrophotometric method for determination of iron(III) and ascorbic acid using betel nut as natural reagent was reported. The efficiency of the system was applied to quantify iron content in rice, vegetable and water samples and quantify ascorbic acid content in pharmaceutical samples. Results obtained by developed method were compared with those achieved from FAAS standards method for determination iron(III) and titration method for determination of ascorbic acid.

1.2 Objectives

1.2.1) To develop green sequential injection analytical method for determination of iron(III) and ascorbic acid using crude extracts from betel nut.
1.2.2) To apply the developed method for analysis of iron(III) and ascorbic acid in real samples.

1.3 Scope of this work

1.3.1) Preliminary study of the complexation reaction between natural reagent extract from betel nut with some metal such as iron(III), iron(II), lead(II), zinc(II), cadmium(II), copper(II), antimony(III), calcium(II), bismuth(III), manganese(II) and aluminum(III).
1.3.2) Investigation on the optimization for betel nut natural reagent extraction such as mass of natural reagent, extraction of solvent and extraction time.
1.3.3) Investigation on the optimum conditions for the quantification of iron(III) using natural reagent such as pH of buffer, concentration of buffer.
1.3.4) Investigation on the optimization conditions for iron(III) determination by sequential injection spectrophotometry such as total volume, sequence order, volume of natural reagent with acetate buffer (R-B), volume of natural reagent, volume of iron(III) standard /or sample, mixing coil length and dispersing flow rate.
1.3.5) Investigation on the optimum conditions for the determination of ascorbic acid such as sequence order, concentration of iron(III) , volume of iron(III), volume of natural reagent, volume of ascorbic acid standard/ or sample, mixing coil length and disperse flow rate .
1.3.6) Method validation in order to evaluate the linearity, limit of detection, limit of quantitation, precision, and accuracy for determination of iron(III) and ascorbic acid.
1.3.7) Application to real samples and comparison the results with standard method.

1.4 Venue of the study

1.4.1) Venue of the study

1.4.1.1) Department of Chemistry, Faculty of Science, Mahasarakham University
1.4.1.2) Scientific Instrument Science Unit, Mahasarakham University

CHAPTER II

LITERATUREVIEW

2.1 Green Analytical Chemistry

Green chemistry was used for chemistry techniques and methodologies that reduce or eliminate volume of products, byproducts, solvents, reagents, etc. It could be product the hazardous to human health or the environment (Keith et al. 2007).

Green analytical chemistry (GAC) emerged from green chemistry in 2000. This new concept has taken a high interest from chemists and it deals with how to make the laboratory practices more environmentally friendly by analytical chemists.

The accuracy, sensitivity, reproducibility, simplicity, speed, and cost reduction are some parameters that any analytical method is considering in development and validation method, while other points towards operator safety and environmental impact are not commonly considered. In some cases, the chemicals used for analysis are more toxic than the species being determined. But current public concern on environmental issues, it became must to adopt an environmentally friendly way (Korany et al. 2017).

A technical term used to call the possibility of utilizing chemical processes in environmentally friendly ways are green chemistry, environmentally benign chemistry, clean chemistry, atom economy, benign by design chemistry, ecological chemistry, environmentally friendly chemistry, sustainable chemistry, ecochemistry (Tobiszewski et al. 2010).

GAC comprises 12 basic principles starting from design and planning, analytical processing development and overall system management including effected use of materials, chemicals, reagents, solvents and energy (Gałuszka et al. 2013). In order to be environmental-friendly are as follows:

1. Direct analytical techniques should be applied to avoid sample treatment.
2. Minimal sample size and minimal number of samples are goals.
3. In situ measurements should be performed.
4. Integration of analytical processes and operations saves energy and reduces the use of reagents.
5. Automated and miniaturized methods should be selected.
6. Derivatization should be avoided.
7. Generation of a large volume of analytical waste should be avoided and proper management of analytical waste should be provided.

8. Multi-analyte or multi-parameter methods are preferred versus methods using one analyte at a time.
9. The use of energy should be minimized.
10. Reagents obtained from renewable source should be preferred.
11. Toxic reagents should be eliminated or replaced.
12. The safety of the operator should be increased.

2.2 Active constituents from medicinal plant

The study of various chemicals found in plants has scope regarding the extraction of important substances such as purification, finding structural formulas, identification of chemicals isolated from plants and analysis for important substances in plants, which found that many substances are include:

2.2.1 Phenolic

Phenolic compounds have possessed one or more aromatic rings with one or more hydroxyl groups. They are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants, with more than 8,000 phenolic structures currently known, ranging from simple molecules such as phenolic acids to highly polymerized substances such as tannins. Plant phenolic is generally involved in defense against ultraviolet radiation or aggression by pathogens, parasites and predators, as well as contributing to plants colors.

2.2.2 Alkaloids

Alkaloids are derived from plant sources; they are basic and contain one or more nitrogen atoms (usually in a heterocyclic ring). Alkaloids are colourless, crystalline, non-volatile, solids. This group also includes some related compounds with neutral and even weakly acidic properties. Alkaloids have a wide range of pharmacological activities including antimalarial (e.g. quinine), antiasthma (e.g. ephedrine), anticancer (e.g. homoharringtonine), cholinomimetic (e.g. galantamine), vasodilatory (e.g. vincamine), antiarrhythmic (e.g. quinidine), analgesic (e.g. morphine), antibacterial (e.g. chelerythrine), and antihyperglycemic activities (e.g. piperine). They have found use in traditional or modern medicine, or as starting points for drug discovery. Other alkaloids possess psychotropic (e.g. psilocin) and stimulant activities (e.g. cocaine, caffeine, nicotine, theobromine), and have been used in entheogenic rituals or as recreational drugs. Alkaloids can be toxic too (e.g. atropine, tubocurarine). Although alkaloids act on a diversity of metabolic systems in humans and other animals, they almost uniformly evoke a bitter taste.

2.2.3 Flavonoids

Flavonoids are found widely throughout the plant and they have a wide range of medicinal uses and actions. They often act as pigments giving a yellow or white color to flowers and fruits. Some flavonoids have anti-viral and anti-inflammatory properties. Flavonoids found in many plants like lemon and buckwheat are known to strengthen capillaries and prevent leakage into tissues.

2.2.4 Polysaccharides

Polysaccharides are found in all plants and comprised of multiple units of sugar molecules linked together. For medicinal purposes, the “sticky” polysaccharides produce mucilage or gums that are commonly found in bark, roots, leaves, and seeds. These sticky polysaccharides are able to soak up large quantities of water and form jelly like masses that can be used to treat dry or irritate tissues such as skin and mucous membranes.

2.2.5 Tannins

Tannins are another major group of polyphenols in our diets and usually subdivided into two groups: (1) hydrolysable tannins and (2) condensed tannins. Hydrolysable tannins are compounds containing a central core of glucose or another polyol esterified with gallic acid, also called gallotannins, or with hexahydroxydiphenic acid, also called ellagitannins. Condensed tannins are oligomers or polymers of flavan-3-ol linked through an interflavan carbon bond. They are also referred to as proanthocyanidins because they are decomposed to anthocyanidins through acid-catalyzed oxidation reaction upon heating in acidic alcohol solutions. Tannins serve as a deterrent to herbivory by insects and grazing animals given that they provide a harsh unpalatable flavor. Tannins are also useful in curing leather because of their tendency to contract and astringe tissues by binding with precipitating proteins. Examples of plants high in tannins include oak bark and black catechu. It also depends on the reaction between tannin and ferric chloride. Is a blue-black-green color to confirm that it actually has tannins.

พหูน ปณ ฑิโต ชีเว

2.3 Techniques for extraction

The extraction is the separation medicinally of active portions of plant using selective solvents through standard procedures. The purpose of all extraction is to separate the soluble plant metabolites, leaving behind the insoluble cellular marc (residue). The initial crude extracts using these methods contain complex mixture of many plant metabolites, such as alkaloids, glycosides, phenolics, terpenoids, tannic acid and flavonoids. Some of the initially obtained extracts may be ready for use as medicinal agents in the form of tinctures and fluid extracts but some need further processing. Several of the commonly used extraction methods are discussed below (Nn, 2015 ; Zhang et al. 2018).

Table 1 Summary of various extraction methods

Method	Solvent	Temperature	Pressure	Time	Volume of organic solvent consumed	Polarity of natural products extracted
Maceration	Water, aqueous and non-aqueous solvents	Room temperature	Atmospheric	Long	Large	Dependent on extracting solvent
Percolation	Water, aqueous and non-aqueous solvents	Room temperature, occasionally under heat	Atmospheric	Long	Large	Dependent on extracting solvent
Decoction	Water	Under heat	Atmospheric	Moderate	None	Polar compounds
Reflux extraction	Aqueous and non-aqueous solvents	Under heat	Atmospheric	Moderate	Moderate	Dependent on extracting solvent
Soxhlet extraction	Organic solvents	Under heat	Atmospheric	Long	Moderate	Dependent on extracting solvent
Pressurized liquid extraction	Water, aqueous and non-aqueous solvents	Under heat	High	Short	Small	Dependent on extracting solvent
Supercritical fluid extraction	Supercritical fluid (usually S-CO_2), sometimes with modifier	Near room temperature	High	Short	None or small	Nonpolar to moderate polar compounds
Ultrasound assisted extraction	Water, aqueous and non-aqueous solvents	Room temperature, or under heat	Atmospheric	Short	Moderate	Dependent on extracting solvent
Microwave assisted extraction	Water, aqueous and non-aqueous solvents	Room temperature	Atmospheric	Short	None or moderate	Dependent on extracting solvent
Pulsed electric field extraction	Water, aqueous and non-aqueous solvents	Room temperature, or under heat	Atmospheric	Short	Moderate	Dependent on extracting solvent
Enzyme assisted extraction	Water, aqueous and non-aqueous solvents	Room temperature, or heated after enzyme treatment	Atmospheric	Moderate	Moderate	Dependent on extracting solvent
Hydro distillation and steam distillation	Water	Under heat	Atmospheric	Long	None	Essential oil (usually non-polar)

2.4 Sample digestion methods

The key difference between dry ashing and wet digestion is depend on the final state of sample that provided dry and an aqueous state (Helen and Christopher, 2017) respectively.

Ashing techniques are very important in analytical chemistry for the analysis of different samples in order to determine their composition. Ash is an inorganic residue that remains after the removal of water and organic matter. Two major processes used in this ashing analysis technique are dry ashing and wet digestion.

2.4.1 Dry Ashing

Dry ashing is an analytical technique which can determine the composition of a sample in dry state. This technique uses very high-temperature muffle furnace for the analysis. And, this furnace should be capable of handling temperatures up to 500-600°C. In this method, water and other volatile materials present in the sample are vaporized upon heating and the organic matter present in the sample is burned in the presence of oxygen in the air. These techniques provide many advantages such as safe, less reagents simultaneously, and specific analyte. But some disadvantages are found such as long time analysis, high costs, and loss of volatile minerals at high temperature such as Cu, Fe, Pb, Hg, Ni and Zn.

2.4.2 Wet digestion

Wet digestion is an analytical technique to determine the composition of a sample in aqueous state. And, this method is mainly used to analyze the composition of a specific mineral in the sample. In this process, the organic matter was broken down and removed from the sample in an aqueous solution. Furthermore, this technique involves heating in the presence of strong acids and oxidizing agents. And, the heating needs to be carried out until the organic matter is completely decomposed. Thus, an only mineral oxide in the solution was eliminated. However, we cannot define a particular time and temperature because it is depend on the type and strength of the acid and oxidizing agent. These techniques provide many advantages such as little loss of volatile minerals occurs because of the lower temperatures used more rapid than dry ashing. But disadvantages are labor intensive, which low sample throughput. Typically, a digestion takes from 10 minutes to a few hours at temperatures of about 350-450^{o}C. The resulting solution can then be analyzed for species of interest.

2.4.3 Microwave digestion

The sample is placed into the microwave muffle furnace and the temperature is ramped up to evaporate the sample, followed by ashing by achieving ashing temperatures. This technique is superior over dry or wet digestion because the time required is drastically decreased, metal losses are less pronounced, and the process is automated by temperature ramps through user-specified method files. Another advantage is the ability to ash larger samples for more accurate results. These techniques provide many advantages such as the short digestion times than regular dry ashing. But disadvantages are is expensive and lower sample throughput than regular dry ashing.

The four major ashing methods described in this chapter are summarized and compared in Table 2.

Table 2 Summary of common ashing methods

Ashing method	Principle	Sources of error	Advantages	Disadvantages	Applications
Dry ashing	Sample heated to very high (500–600 °C) temperature. All organic matter incinerated. Remaining inorganic material quantitated gravimetrically	Microelement contamination (from grinder or water used to clean crucibles). Sample loss during pre-ash drying step. Volatilization of some elements. Incomplete combustion	Can analyze many samples at once. Requires little technician time. Safe. No blanks needed	Slow (takes 12–18 h); some minerals volatilized. Minerals difficult to resolubilize	Total ash content for proximate analysis. May be used as preparation for specific mineral analysis
Wet digestion	Organic matter oxidized using acids and oxidizing agents, leaving inorganic matter	Microelement contamination. Must run blanks to correct for organic matter in acid and oxidizing agent. Sample loss may occur due to spattering	Shorter time (~2 h) than dry ashing. Minerals stay in solution. Little or no volatilization	Requires constant attention. Lower sample throughput than dry ashing. Can be dangerous. Use of strong acids and oxidizers. Sample loss due to spattering	Ashing of samples prior to mineral analysis for official analyses
Microwave digestion (dry)	Microwave energy heats sample to very high temperatures. Incinerates organic matter. Leaves inorganic matter to be quantitated gravimetrically	Microelement contamination (from grinder or water used to clean crucibles). Sample loss during pre-ash drying step. Volatilization of elements. Incomplete combustion	More rapid (~30 min) than regular dry ashing	Costly. Lower sample throughput than regular dry ashing. Some minerals volatilized. May be hard to resolubilize	Determine total ash content for determination of proximate composition or for quality control purposes
Microwave digestion(wet)	Microwave energy and acid (and sometimes oxidizing agent) are used to oxidize and incinerate organic matter, leaving inorganic matter	Microelement contamination. Must runs blanks to correct for any organic matter in acid and oxidizing agent	Takes less time (~30 min) than regular wet ashing. Minerals stay in solution. Little or no volatilization	Expensive. Can handle fewer samples per run than standard wet or dry ashing procedures	Rapid ashing of samples prior to mineral analysis by rapid or official methods

2.5 Betel nut

Figure 1 Cross-section of a betel nut

The medicinal plant used in this research is betel nut. Important taxonomy information of medicinal plants was presented of the following:

Kingdom	Plantae
Scientific name	*Areca catechu* Linn.
Family	Arecaceae
Common name	Betel Nut, Betel Palm

Betel nut is widely cultivated in several South Asian and Southeast Asian countries including India, China, Bangladesh, Indonesia, Myanmar, Thailand, Malaysia, Vietnam, Philippines and etc. Its fruit or seed is called areca nut or betel nut. It has characteristic astringent and slightly bitter in taste (Wang et al. 1997) . Betel nut is one of popular traditional herbal medicine used in Thailand. The activities of areca seeds are anthelmintic, antifungal, antibacterial, antiinflammatory, antioxidant, insecticide, and lavicidal. The seed contains sugars, lipid (glyceride of lauric, myristic and oleic acid), condensed tannins (phlobatannin, catechin), polyphenolics and alkaloids (arecoline, arecaidine, guvacine and guvacoline) (Rathod, Shivaprasad, 2015). The contents of total phenolics and condensed tannin increased in a maturity dependent manner are reported in the Table 3.

Table 3 Contents of total phenolics and condensed tannins in betel nut

Sample	Content (mg/g of fresh wt.)	
	total phenolics	**tannin[b]**
root	17.14±0.33[ac]	18.05±6.61[a]
leaf	5.49±0.36[d]	3.67±0.66[c]
spike	4.72±0.90[d]	1.78±0.47[e]
vein	2.41±1.31[e]	1.33±1.03[e]
tender shoot	0.58±0.01[f]	0.85±0.16[e]
calyx	3.52±0.51[d]	1.22±0.36[e]
flower	3.83±0.81[d]	2.15±0.45[d]
unripe fruit (2 cm)	5.78±0.86 [d]	9.03±1.90[b]
unripe fruit (3 cm)	9.28±0.65[c]	7.84±0.95[b]
ripe fruit	12.63±0.41[b]	9.85±0.88[b]
upside-down fruit	8.79±0.32[c]	8.32±0.41[b]

[a] Units: mg of gallic acid equiv/g of fresh wt.
[b] Units: mg of cateching equiv/g of fresh wt.
[c] Data bearing different superscript letters in the same column are significantly different ($p < 0.05$).
[d] Unripe areca fruit of 2 cm in length.
[e] Unripe areca fruit of 3 cm in length (commercial size).

As illustrated in Table 3, the contents of total phenolics and tannin were very rich in root (17.14 and 18.05 mg/g of fresh wt.) followed by areca fruit, leaf, spike, vein, and tender shoot, respectively. Edible tender shoot contained few total phenolics and tannin (0.58 and 0.85 mg/g of fresh wt.).

2.6 Sequential injection analysis

SIA was developed by J. Ruzicka and G. Marshall as an alternative to FIA (Ruzicka and Marshall, 1990). SIA has proved that its scope departs markedly from that of the earlier technique. Figure 2 shows a schematic depiction of a typical SIA system.

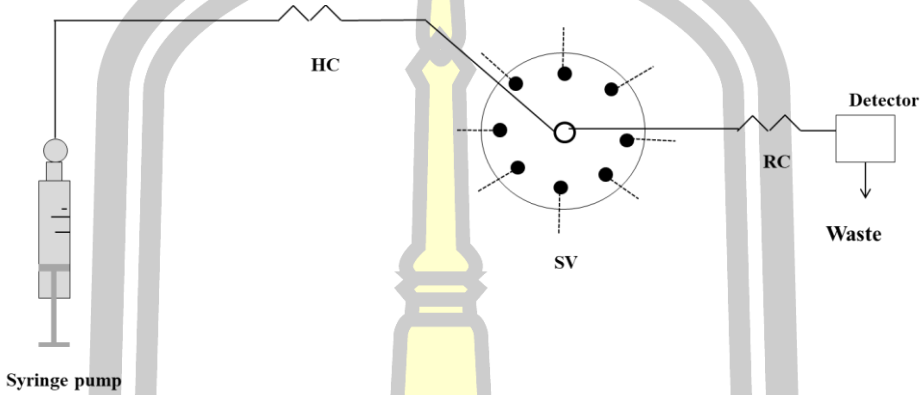


Figure 2 Schematic depiction of a typical SIA system. HC: holding coil, RC: reaction coil, SV: selection valve

The principal components of the sequential injection analyzer are pump or syringe, selection valve, detector, holding coil, reaction coil and computer. The lateral ports of the valve are connected to the recipients that contain the sample, the carrier and the reagents required for the analytical determination, and to the detector.

The process involves the following steps. First, the reagents are aspirated through the selected valve. The syringe aspirates the carrier, the sample and the reagents. Next, the syringe pump pushes the reagents towards the detector. In this step, the direction of flow is changed. Finally, the detector provides information about the measurements registered in a period of time and a prefixed range of wavelengths (absorbances in our case) and once the sample has passed though the detector, it is expelled as waste.

2.7 Fiber optical spectrometer

2.7.1 Basic principles

The basic principle of light transport through an optical fiber is total internal reflection. This means that the light within the numerical aperture of a fiber (NA = input acceptance cone) will be reflected and transported through the fiber. The size of the numerical aperture depends on the materials used for core and cladding. Two basic types of silica fibers can be distinguished; single-mode and multi-mode fibers, depending on the propagation state of the light, traveling down the fiber. For most spectroscopic applications multi-mode fibers are used. Multi-mode fibers can be divided into 2 subcategories, step-index and graded-index. A relatively large core and high NA allow light to be easily coupled into the fiber, which allows the use of relatively inexpensive termination techniques. Step-index fibers are mainly used in spectroscopic applications. Graded-index multimode fibers have a refractive index gradually decreasing from the core out through the cladding. Since the light travels faster in material with lower refractive index, the modal dispersion (amount of pulse-spreading) will be less. These graded-index fibers are mainly used in telecommunication application, where dispersion at long distance (2-15 km) plays an important role.

A fiber-optic spectrometer is an instrument used to measure properties of light (often using a light source, a fiber-optic cable and software) over a specific part of the electromagnetic spectrum. In the case of fiber-optic spectroscopy, the focus lies on ultraviolet radiation, the visible spectrum, and near-infrared radiation, as is pictured in Figure 3.

Figure 3 Fiber-optic spectrometer instruments

Spectrometers generally consist of an entrance slit, which is where the light enters the spectrometer via a fiber-optic cable, a collimating mirror that causes the light to concentrate into a parallel beam, a grating (refractive element), focusing optics that deflect the refracted light to reach the electronics and a detector that converts the measured light into comprehensible data in our software. Spectrometers is optimized with light traps and filters to make sure the detector captures the minimal amount of stray light to make your measurement as accurate as possible.

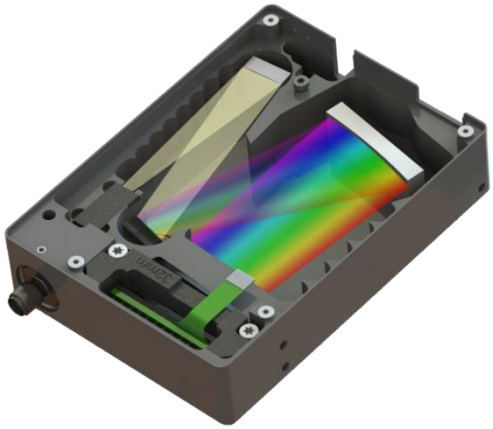


Figure 4 Inside of a spectrometer

(https://www.avantes.com/products/what-is-a-spectrometer)

2.8 Literature Review

2.8.1 The use of plant extracts as reagents in chemical analysis

Some studies (Grudpan et al. 2010; Hartwell, 2012) have been done on the use of plant extracts as a chemical analysis reagent, and it can be divided into two groups:

2.8.1.1 Natural pH indicator

The researchers worked using a different of plants such as beetroot (Grudpan et al. 2011), pea flower (Grudpan et al. 2011), orchid (Grudpan et al. 2011), amaranth (Uede et al. 2011). al., 2010), bacheller batton (Uede et al. 2010) extracts vital compounds to use as pH indicators, mainly betacyanin (Grudpan et al. 2011; Uede et al. 2010), anthocyanin (Grudpan et al. 2011; Uede et al. 2011), which can change color or have the ability to absorb light as the pH changes.

2.8.1.2 Natural chromogenic reagent

The researchers were carried out by extracting a different of plants with different active ingredients to form complex compounds with some metals such as guava leaf (Settheeworrarit et al. 2005), green tea (Pinyou et al. 2010), Ma kham pom (Jaikrajang et al. 2018), Smilax china root (Ganranoo et al. 2019) as an alternative natural reagent for quantification of iron. Indian almond (Insain et al. 2013), heartwood of sappan wood (Siriangkhawut et al. 2016), indian mulberry root (Tontrong et al. 2012), Pisum sativum (Gao et al. 2006) has been applied for the determination of aluminium**.**

The use of natural reagent extracted from plants in chemical analysis by flow-based analysis for determination of metal ions is shown in Table 4. Although, these obstacles, novel methods which are low cost, rapid and use fewer chemicals.

Table 4 The use of natural reagent extracted from plants in chemical analysis by flow- based analysis for determination of metal ions

Year	Authors	Natural chromogenic reagent extracted	Analysis	Sample	LOD
2005	Settheeworrarit et al *	guava leaf	iron	Tap water	-
2006	Gao et al.	Pea	mercury	wastewater	1.3 µM
2010	Pinyou et al.	green tea	iron	Pharmaceutical	0.05 mg L^{-1}
2012	Tontrong et al.	Indian mulberry root	aluminium	Tea	0.05 mg L^{-1}
2013	Insain et al.	Indian almond	aluminium	Wastewater	0.8 mg L^{-1}
2016	Siriangkhawut et al.	Heartwood of sappan wood	aluminium	Pharmaceutical /Tap water/ Beverage	0.021 mg L^{-1}
2018	Jaikrajang et al.	Ma-kham-pom	iron	Pharmaceutical /Tap water/ground water	0.31 mg L^{-1}
2019	Ganranoo et al.	Smilax china root	iron and manganese	Groundwater	0.05 mg L^{-1}

*Note: No reports of limit of detection (LOD)

2.8.2 Analytical techniques for determination of iron

Various techniques have been studied and developed for iron assay. The techniques have been reported for the determination of iron content as presented in Table 5.

Table 5 Literatures the analytical techniques for determination of iron

Year	Authors	Analytical techniques
2002	Sreenivasa et al.	Inductively coupled plasma-atomic emission spectrometry
2003	Aleixo et al	Graphite furnace atomic absorption spectrometry
2016	Leao et al.	
2019	Adolfo et al.	
2003	Kehm et al.	Inductively coupled plasma-mass spectrometry
2006	Dufailly et al.	
2010	Aydin et al.	
2010	Ghoneim et al	Square-wave adsorptive cathodic stripping voltammetry
2008	dos Santos et al.	Flame atomic absorption spectrometry
2008	Silva et al.	
2012	Rojas et al.	
2016	Pourjavid et al.	

Although these methods are capable of achieving highly sensitive detection, identification and quantification of heavy metals. But it also requires support from well-equipped laboratories and skilled operators, which can be expensive. Thus, alternative and inexpensive methods are preferred for determining iron.

Spectrophotometric methods are widely used for iron measurements due to their simplicity, speed, low cost and wide applications. There are many spectrophotometric methods reports for determination of iron by using different reagents to determine iron content such as 1,10-phenanthroline (Tesfaldet et al. 2004) squaric acid (1,2-dihydroxy-3,4-diketocyclobutene) (Stalikas et al. 2003), 2-(5-bromo-2-pyridylazo)-5-[N-n-propyl-N-(3-sulfopropyl)amino]aniline (5-Br-PSAA) (Ohno et al. 2006), thiocyanate (Verma et al. 2017), 8-hydroxyquinoline (Adebayo et al. (2011) and 2-(5-bromo-2- pyridylazo)-5-diethylaminophenol (5-Br-PADAP) (Filik et al. 2012). It can be used to analyze iron content in several of samples, including environmental (González et al. 2017) drugs (Balcerzak et al. 2008), multivitamin drugs (Tesfaldet et al. 2004) and food (Moghadam et al. 2016; Peng et al. 2015). The spectrophotometric methods using commercial available and synthetic complexing

reagents for determination of iron are shown in Table 6. These reagents may themselves be toxic and have health impacts on humans.

Table 6 Literatures the spectrophotometric methods using complexing reagents for determination of iron

Year	Authors	Reagent
2003	Stalikas et al.	1,2-Dihydroxy3,4-Diketocyclo-Buten (Squaric Acid)
2004	Tesfaldet et al.	1,10-phenanthroline
2006	Ohno et al.	2-(5-bromo-2-pyridylazo)-5-[N-n-propyl-N-(3 sulfopropyl)amino]aniline (5-Br-PSAA)
2009	Sharma et al.	2,6-bis(1- hydroxy-2-naphthylazo)pyridine (PBN)
2011	Adebayo et al.	8-hydoxyquinoline
2012	Filik et al.	2-(5-bromo-2-pyridylazo)-5-diethylaminophenol (5-Br-PADAP)
2017	Verma et al.	Thiocyanate

2.8.3 Ascorbic acid

Ascorbic acid (vitamin C) is a water-soluble vitamin. It occurs as a white or slightly yellow crystal or powder with a slight acidic taste. It is an antiscorbutic product. On exposure to light, it gradually darkens. In the dry state, it is reasonably stable in air, but in solution it rapidly oxidizes. Ascorbic acid is freely soluble in water; sparingly soluble in alcohol; insoluble in chloroform, ether, and benzene. The chemical name of ascorbic acid is L-ascorbic acid. The empirical formula is $C_6H_8O_6$, and the molecular weight is 176.13. The synthesis of ascorbic acid was achieved by Reichstein in 1933, followed by industrial production of ascorbic acid two years later by Roche. The ultimate raw material for the production of ascorbic acid is corn or wheat. This is converted via starch to glucose by specialist companies, and then to sorbitol. Ascorbic acid produces the pure final products from sorbitol in a series of biotechnical, chemical processing and purification steps.

Ascorbic acid is probably one of the most highly well known. Furthermore, people have become more aware to the importance of vitamin C. Hence, this causes the global market flooded with ascorbic acid fortified foods (Arya et al.2000). The term of ascorbic acid is used as generic term for all compounds exhibiting qualitatively the biological activity.

2.8.3.1 Analytical techniques for determination of ascorbic acid

Varieties of analytical methods have been studied for determination of ascorbic acid. The techniques have been reported for the determination of ascorbic acid as presented in Table 7.

Table 7 Literatures the analysis techniques for determination of ascorbic acid

Years	Authors	Analytical techniques
1996	Zhang and Qin	Chemiluminescence
1994	Ross	High performance liquid chromatography
2015	Zuo et al.	
2005	Silva	Gas chromatography
2008	Danet et al.	Voltammetry
2005	Pavan et al.	Amperometric sensor
2001	Jiang et al.	Atomic absorption spectroscopy

Most of these methods have a prior separation procedure of the product reduced by ascorbic acid and are unsuitable for routine application in terms of the rate of analysis. At present, spectrophotometry is the most commonly employed methods for determine ascorbic acid.

Different oxidation-reduction systems for spectrophotometric determination of ascorbic acid have been proposed as Cu(II) and alizarin red S (Kukoc-Modun et al. 2012), 2,4-dinitrophenylhydrazine (Khan et al 2006) , Folin–Ciocalteu (Olgun et al. 2014), Potassium Iodate (Qureshi et al. 1991), Prussian Blue (Nóbrega et al. 2010), Cu(II) and Neucoproine (Shrivas et al. 2005, 2,4,6-tripyridyl-s-triazine (Kukoc-Modun et al. 2012), and 11-Molybdobismuthophosphate (Vishnikin et al. 2010). These reagents may themselves be hazardous, expensive and unfriendly to the environment shown in Table 8. To overcome these obstacles, therefore, develop novel methods of analysis for determination of ascorbic acid use natural reagent was proposed. The new spectrophotometric method and alternative techniques for the determination of ascorbic acid could be developed.

Table 8 Literatures the spectrophotometric method for determinations of ascorbic acid

Years	Authors	Reagent
1991	Olgun et al.	Folic-Ciocalteu
1991	Qureshi et al.	Potassium Iodate
2005	Shrivas et al.	Cu(II) and Neucoproine
2006	Khan et al.	2,4-dinitrophenylhydrazine
2010	Nóbrega et al.	Prussian blue
2010	Vishnikin et al.	11-Molybdobismuthophosphate
2012	Kukoc-Modun et al.	Cu(II) and alizarin red S
2012	Kukoc-Modun	2,4,6-tripyridyl-s-triazine

CHAPTER III
MATERIALS AND METHODS

3.1 Chemicals and reagents

All chemicals and reagents used in this work listed in Table 9 were analytical regent grade and they were used without further purification.

Table 9 List of chemicals used in this work

Chemicals	Formula	Grade	Company	Country
Stock solution of iron(III) 1000 mg L^{-1}	-	AR	Merck	Germany
Stock solution of copper (II) 1000 mg L^{-1}	-	AR	Merck	Germany
Stock solution of aluminum(III) 1000 mg L^{-1}	-	AR	Merck	Germany
Stock solution of zinc(II) 1000 mg L^{-1}	-	AR	Merck	Germany
Stock solution of iron (II) 1000 mg L^{-1}	-	AR	Merck	Germany
Stock solution of cadmium(II) 1000 mg L^{-1}	-	AR	Merck	Germany
Stock solution of antimony(III) 1000 mg L^{-1}	-	AR	Merck	Germany
Stock solution of calcium(II) 1000 mg L^{-1}	-	AR	Merck	Germany
Stock solution of bismuth(III) 1000 mg L^{-1}	-	AR	Merck	Germany
Stock solution of manganese(II) 1000 mg L^{-1}	-	AR	Merck	Germany
Stock solution of lead(II) 1000 mg L^{-1}	-	AR	Merck	Germany
Sodium acetate 3- hydrate	$CH_3COONa.3H_2O$	AR	Ajax Finechem	New Zealand
Nitric acid	HNO_3	AR	Merck	Germany
Acetone	C_3H_6O	AR	Merck	Germany
Ethanol	C_2H_5OH	AR	Merck	Germany
Methanol	CH_3OH	AR	Merck	Germany
Acetonitrile	CH_3CN	HPLC	Merck	Germany
Acetic acid	CH_3COOH	AR	Merck	Germany
L-ascorbic acid	$C_6H_8O_6$	AR	Chem-Supply	Australia
DI water	H_2O	-	Milli-Q, Millipore	United States

AR grade chemical means analytical reagent

HPLC grade chemical means high performance liquid chromatography

3.2 Instrument and apparatus

Apparatus used in this research were presented in Table 10.

Table 10 List of the apparatus used in this work

Apparatus	Model	Company	Country
Fiber optic spectrometer	AVANTES	-	Netherlands
UV-Vis spectrophotometer	Cary 60	Agilent	United States
Flame atomic absorption spectrometer	240FS AA	Agilent	United States
Perkin-Elmer spectrophotometer	Spectrum GX	Lambda 25	United States
Furnace	CWF1200	Carbolite	United Kingdom
Syringe pump	XL-3000	Cavro	United States
10-port selection valve	TX	Valco Instrument	United States
Flow through cell	170-700-QS	Hellma	Germany
Centrifuge	1040series	Labquip	United Kingdom
pH meter	713	Metrohm	Switzerland
Water bath	TW12	Julabo	Germany
Vortex mixer	232	Fisher Scientific	United States
Micropipette 100 µL	l25291B	Eppendorf	Germany
Micropipette 1000 µL	l19716B	Eppendorf	Germany
Electronic balance	PA 214	Ohaus	United States
Blender	EBR	Electrolux	Thailand
Hotplate and stirrer	-	-	-
Other experimental equipment*	-	-	-

Other experimental equipment* such as volumetric flask, beakers, test tube rack, watch glass, graduated pipette, wash bottle, glass funnel etc.

3.3 Experimental

3.3.1 Preparation of standard solution

3.3.1.1 Stock standard solution of iron(III) 100 mg L^{-1}

A 100 mg L^{-1} iron(III) standard stock solutions was prepared by pipette 1 mL of 1000 mg L^{-1} of iron(III) into 10 mL volumetric flask. Then, the final volume was adjusted to 10 mL with deionized water.

3.3.1.2 Stock standard solution of iron(III) 10 mg L^{-1}

A 10 mg L^{-1} iron(III) standard stock solutions was prepared by pipette 1 mL of 100 mg L^{-1} of iron(III) into 10 mL volumetric flask. Then, the final volume was adjusted to 10 mL with deionized water.

3.3.1.3 Stock standard solution of ascorbic acid 1000 mg L^{-1}

Stock standard solution of ascorbic acid was prepared daily by weighing 0.025 g ascorbic acid in 25 mL volumetric flask and adjusted volume to 25 mL with deionized water. Working solution of ascorbic acid was prepared by appropriate dilution of stock ascorbic acid solution by deionized water.

3.3.2 Preparation of buffer solution

Buffer solution was prepared by mixing appropriate amount of sodium acetate and acetic acid for pH 4.5-5.5, disodium hydrogen phosphate and sodium dihydrogen phosphate monohydrate for pH 6-8, ammonia and ammonium chloride for pH 8.5-10. The required pH was performed by adjusting with sodium hydroxide solution.

3.3.3 Preparation of different concentration of acetate buffer pH 5.5

Acetate buffer pH 5.5 in different concentration was prepared follow Table 11.

Table 11 Preparation of acetate buffer concentration

Concentration of acetate buffer	Weight of $CH_3COONa.3H_2O$ (g)	Volume of CH_3COOH (mL)
0.05	0.2878	0.02
0.1	0.5756	0.04
0.3	1.7269	0.13
0.7	4.0293	0.31
1	5.7562	0.44

3.3.4 Preparation of natural reagent

The seed of betel nut more than 5.5 kg was purchased from a local supermarket in Roi-Et, Thailand. All samples were chopped into small pieces and dried in an oven 60 °C for 24 h. After that, dried reagent was homogenized utilizing cooking blender (Electrolux, Thailand). A physical characteristic of dried betel nut and homogenies betel nut was shown in Figure 5. Then, reagent was stored in a desiccator.

Figure 5 Physical characteristics of betel nut

3.3.5 Extraction method

Natural reagent was prepared by transferring 0.5 g of betel nut powder to a 250 mL beaker. Then, natural reagent powder was mixed with 100 mL of deionized water. After that, heated and stirrer on a hotplate for 20 min. After cooling, the extract solution was transferred into a 15 mL centrifuge tube, then centrifuge at 6,000 rpm for 10 min. The extract was filtered through a filter paper (Whatman® No.1) and adjusted to a volume of 100 mL with deionization water.

3.4 Synthesis of the iron- betel nut complex

The iron(III)-betel nut complex was synthesized by mixing 1 mL of the betel nut extract solution with 1 mL of iron(III) 1000 mg L^{-1} and 5 mL of the acetate buffer at pH 5.5 . The final volume was adjusted to 10 mL with deionized water. The resulting dark violet solids were precipitated. After that, the solution was filtrated and then dried in an oven at 80 °C for 24 h. The infrared spectrum of iron (III)-betel nut complex were recorded using pellet technique as KBr disks on Perkin-Elmer spectrophotometer between 4000 and 500 cm^{-1}. The infrared spectra was comparison with the iron(III)-tannic acid complex.

3.5 Preliminary study

3.5.1 Study on complexation of natural reagent with some metal ions

The mixing of various ions at 10 mg L^{-1} of iron(III), iron(II), lead(II), zinc(II), cadmium(II), copper(II), antimony(III), calcium(II), bismuth(III), manganese(II) and aluminum(III) were mixed with 1 mL natural reagent, 5 mL acetate buffer pH 5.5 in the 10 mL of volumetric flask. Then, the absorption spectrum of solution was measured by spectrophotometer from 350-800 nm.

3.5.2 Study of the maximum absorption spectra

The maximum wavelength of complexing products was evaluated from absorption spectra obtained from 3.5.1. The wavelength providing the highest absorption signal was selected for next studied parameters.

3.5.3 Optimization of natural reagent extraction

3.5.3.1 Type of solvent for extraction of natural reagent

The natural reagent solution from the betel nut was obtained by stirring with different type of solvent as deionized water, methanol, acetone, acetonitrile, ethanol, 50% ethanol, and heat deionized water. After that, the crude extract solution obtained from each solvent was tested with 10 mg L^{-1} of iron(III) at pH 5.5 in a 10 mL volumetric flask. Using the volumes of different solutions as shown in Table 12, the absorbance at a wavelength of 565 nm was compared.

Table 12 Volumes for the preparation of solvents

	Type of solvent	Volume (mL)			Total volume (mL)
		Betel nut extract solution	1 mol L^{-1} acetate buffer pH 5.5	100 mg L^{-1} iron(III)	
Without heat	Acetone	1	5	1	10
	Acetonitrile	1	5	1	10
	Methanol	1	5	1	10
	Ethanol	1	5	1	10
	50% v/v Ethanol	1	5	1	10
	Deionized water	1	5	1	10
Heat	Deionized water	1	5	1	10

3.5.3.2 The effect of betel nut mass

The effect of betel nut mass was studied at different of 0.1 - 5.0 g. The betel nut extract was prepared by using deionized water with heat (hot water) as extraction solvent according to the method in Section 3.3.5. The betel nut extracts obtained from different weights were prepared. After that, the betel nut extract was mixed with iron(III) at a concentration of 10 mg L^{-1} , pH 5.5 in a 10 mL volumetric flask using different volumes as shown in Table 13. The absorbance values were compared at the wavelength of 565 nm.

Table 13 Volumes for the preparation of mass of betel nut

Mass of betel nut (g)	Volume (mL)			Total volume (mL)
	Betel nut extract solution	1 mol L^{-1} acetate buffer pH 5.5	100 mg L^{-1} iron(III)	
0.1	1	5	1	10
0.3	1	5	1	10
0.5	1	5	1	10
1	1	5	1	10
2	1	5	1	10
3	1	5	1	10
4	1	5	1	10
5	1	5	1	10

3.5.3.3 The effect of extraction time

The extraction time was studied at different intervals: 10-60 minutes. The crude extract from betel nut was prepared according to the method in Section 3.3.5. The betel nut extract obtained from the preparation at different times was taken. After that, the betel nut extract was mixed with iron(III) at a concentration of 10 mg L^{-1}, pH 5.5 in a 10 mL volumetric flask using different volumes as shown in Table 14. The absorbance values were compared at the wavelength of 565 nm.

Table 14 Volumes for the preparation of different time extraction

Time (min)	Volume (mL)			Total volume (mL)
	Betel nut extract solution	1 mol L^{-1} acetate buffer pH 5.5	100 mg L^{-1} iron(III)	
10	1	5	1	10
20	1	5	1	10
30	1	5	1	10
40	1	5	1	10
60	1	5	1	10

3.5.4 Optimum conditions for the quantification of iron(III) using natural reagent

3.5.4.1 The effect of pH

Betel nut extract was mixed with iron(III) at a concentration of 10 mg L^{-1} in different buffer at pH between 4.5-10 using the volume of each solution as shown in Table 15. The prepared solution was then taken to measure the absorbance at wavelength 565 nm.

Table 15 Volumes for the preparation of buffer solution

pH	Volume (mL)			Total volume (mL)
	Betel nut extract solution	1 mol L^{-1} acetate buffer pH 5.5	100 mg L^{-1} iron(III)	
4.5	1	5	1	10
5	1	5	1	10
5.5	1	5	1	10
6	1	5	1	10
6.5	1	5	1	10
7	1	5	1	10
7.5	1	5	1	10
8	1	5	1	10
8.5	1	5	1	10
9	1	5	1	10
9.5	1	5	1	10
10	1	5	1	10

3.5.4.2 The effect of acetate buffer concentration

The effect of buffer concentration on the absorption signal of iron determination were studied at the buffer concentration of 0.05- 1 mol L^{-1}. As shown in Table 16, the prepared solution was then taken to measure the absorbance at wavelength 565 nm.

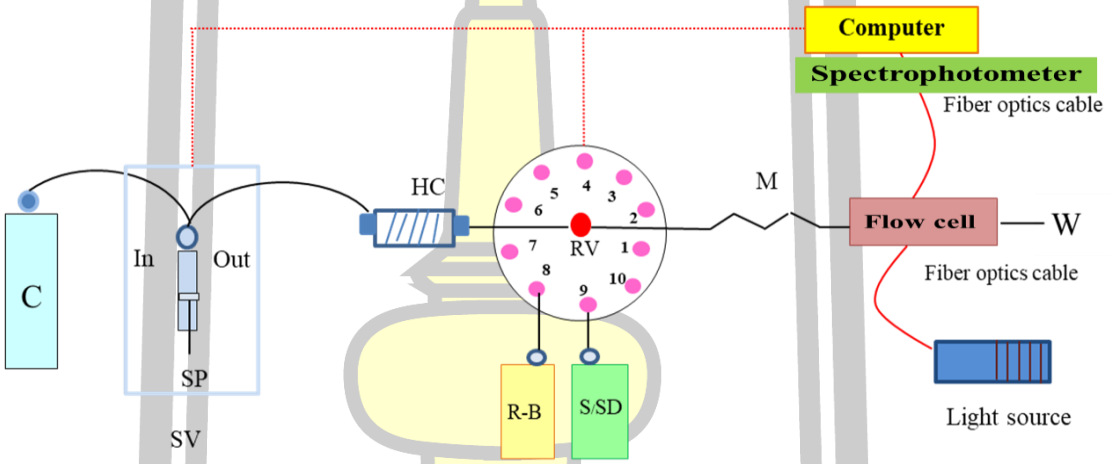
Table 16 Volumes for the preparation of concentration of buffer

Concentration of buffer (mol L^{-1})	Volume (mL)			Total volume (mL)
	Betel nut extract solution	1 mol L^{-1} acetate buffer pH 5.5	100 mg L^{-1} iron(III)	
0.05	1	5	1	10
0.1	1	5	1	10
0.3	1	5	1	10
0.5	1	5	1	10
0.7	1	5	1	10
1	1	5	1	10

3.5.5 Sequence injection analysis (SIA) for iron assay

An in-house assembled sequential injection analysis (SIA) system utilized in this research work was illustrated in Figure 6 (a). This SIA system consisted of 5.0 mL syringe pump Model XL-3000, 10-port selection valve, reaction coil (PTFE tubing i.d 0.5 mm., long 1 m) and a holding coil (HC; 250 cm PTFE tubing; OD 1/16″, ID 0.03″). A fiber optic spectrometer with a flow through cell (Quartz, 10 mm path length, 80 µL internal volume was used for spectrophotometric determination. The SIA system was controlled by in-house created software based on Visual Basic 6.0. A personal computer with Avasoft 8.0 software was used for signals acquisition.

(a)



(b)

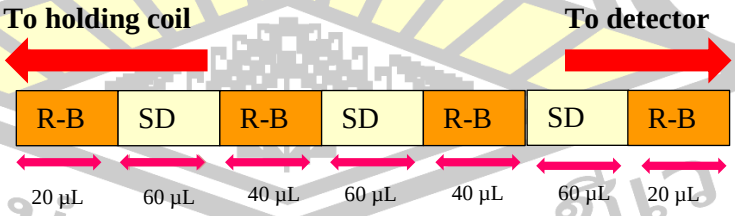


Figure 6 (a) SIA system for determination of iron (III); C, carrier (deionization water); SP, syringe pump; SV, switching valve; HC, holding coil; M, mixing coil; RV, ten-port rotary selection valve; R-B, natural reagent with buffer solution (pH 5.5); SD, Iron (III) standard solution; S, sample solution; W, waste and **(b)** sequence order for aspiration of solution in the holding coil

3.5.5.1 Optimization conditions for iron determination by sequential injection spectrophotometry

3.5.5.1.1 SIA procedures for determination of iron(III)

The operation steps of the developed method for the determination of iron(III) are shown in Table 17. The operation steps was initiated by filling the holding coil (HC), flow through cell and PTFE tubing with a carrier solution by deionized water. Tubing connected was filled with reagents and standard, their respective solution. Subsequently, the natural reagents (R-B solution; via valve position 8 in steps 2, 4, 6, 8) and a series of standard iron(III) solutions or sample (via valve position 9 in steps 3, 5, 7) were sequentially aspirated into a holding coil according to the solution sequence order illustrated in Figure 6 (b). The total volume of natural reagent and sample per an analysis was 120 and 180 µL, respectively. After that, the aspirated zones of sample and reagents were dispensed into the reaction coil, and flowed to the detection flow cell for measurement of the absorbance at 565 nm (step 9).

Table 17 Operation procedure of SI system for determination of iron(III) with natural reagent extract from the seed of betel nut

Step	Valve position	Valve of pump	Flow rate/µL s^{-1}	volume/ µL	Descriptions
1	–	In	100	1500	Aspirate water from carrier solution
2	8	Out	70	20	Aspirate R-B solution
3	9	Out	50	60	Aspirate standard of sample solution
4	8	Out	70	40	Aspirate R-B solution
5	9	Out	50	60	Aspirate standard of sample solution
6	8	Out	70	40	Aspirate R-B solution
7	9	Out	50	60	Aspirate standard of sample solution
8	8	Out	70	20	Aspirate R-B solution
9	1	Out	50	1800	Dispense all solution through detector to waste

3.5.5.1.2 The effect of sequence order

The effects of sequent order for determination of iron were being designed 5 segments as presented in Table 18. The volume of reagent and iron were fixed at 150 µL for total 300 µL. Iron(III) at 10 mg L^{-1} was utilized through optimum conditions.

Table 18 Sequent order for determination of iron(III) standard

Sequence No.	Sequence order	volume (µL)
1	R-B/SD	150/150
2	R-B/SD/R-B	75/150/75
3	R-B/SD/R-B/SD/R-B	50/75/50/75/50
4	R-B/SD/R-B/SD/R-B/SD/R-B	25/50/50/50/50/50/25
5	R-B/SD/R-B/SD/R-B/SD/R-B/SD/R-B/SD/R-B/SD	(25/25) *6

3.5.5.1.3 The effect of natural reagent volume adjusts by acetate buffer pH 5.5 (R-B)

The effect of natural reagent volume adjusts by acetate buffer pH 5.5 on the absorption signal of iron determination were studied by mixing natural extraction solution between 0.5- 20 mL with acetate buffer. Then, the solution was adjusted to 25 mL with acetate buffer pH 5.5.

3.5.5.1.4 The effect of aspirate volume of R-B solution

The effect of aspirate volume of natural reagent on the absorption signal of iron determination was studied in the range 30-240 µL.

3.5.5.1.5 The effect of aspirate volume of iron standard /sample solution

The effect of aspirate volume of iron standard/sample on the absorption signal of iron determination was studied at 60-270 µL.

3.5.5.1.6 The effect of mixing coil length

The effect of mixing coil on the absorption signal of iron determination was studied study at the mixing coil of 0-150 cm.

3.5.5.1.7 The effect of dispensing flow rate

The effect of dispensing flow rate on the absorption signal of iron determination was studied at the dispensed flow rate of 30 -110 µL s^{-1}.

3.5.6 Optimum conditions for the quantification of ascorbic acid

An in-house assembled sequential injection analysis (SIA) system utilized in this research work was illustrated in Figure 7 (a)

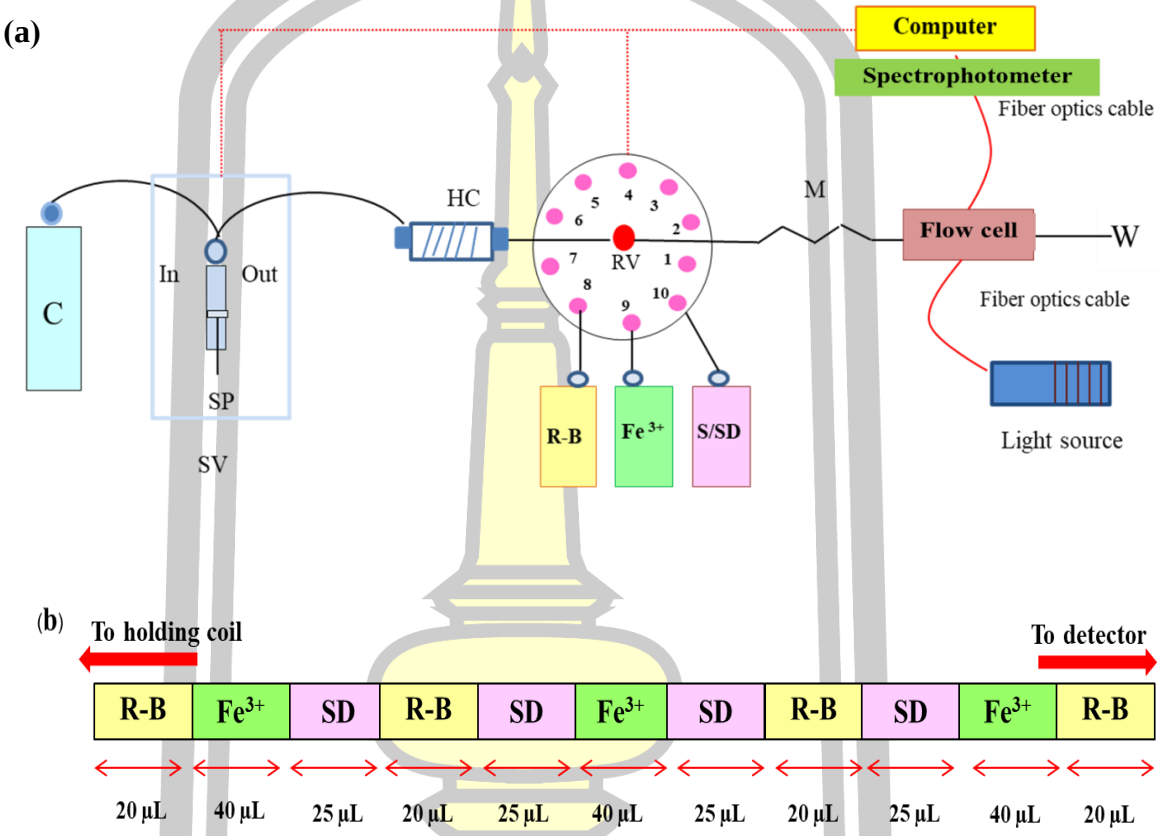


Figure 7 (a) Sequential injection analysis manifold configuration. C, carrier (deionization water); SP, syringe pump; SV, switching valve; HC, holding coil; RV, ten-port rotary selection valve; R-B, natural reagent with buffer solution (pH 5.5), S: sample or standard solution (ascorbic acid): M, mixing coil. W, Waste and **(b)** sequence order for aspiration of solution in the holding coil

3.5.6.1 SIA procedures for determination of ascorbic acid

The sequence of operations for the developed method is shown in Table 19. The sequence was initiated by filling the holding coil (HC) with the carrier solution (DI water). After that, the tubing was filled with R-B solution, iron(III) solution and standard/sample solution respective. Sequentially, the R-B solution; (via valve position 8 in steps 2, 5, 9, 12), the iron(III) solution; (via valve position 9 in steps 3, 7, 11) and a series of standard ascorbic acid solutions or sample (via valve position 10 in steps 4, 6, 8, 10) were aspirated into the holding coil according to the sequence order of solutions shown in Figure 7 (b). The total volume of R-B solution, iron(III) solution and sample was 80, 120 and 100 µL, respectively. Then, 300 µL of the solution was dispensed into the reaction coil, and flow to the detection flow cell to measure the absorbance at wavelength of 565 nm (step13).

Table 19 The procedure of sequential injection system for determination of ascorbic acid

Step	Valve position	Valve of pump	Flow rate/µL s^{-1}	volume/ µL	Descriptions
1	–	In	100	1500	Aspirate water from carrier solution
2	8	Out	70	20	Aspirate R-B solution
3	9	Out	50	40	Aspirate iron(III)
4	10	Out	50	25	Aspirate standard or sample solution
5	8	Out	70	20	Aspirate R-B solution
6	10	Out	50	25	Aspirate standard or sample solution
7	9	Out	50	40	Aspirate iron(III)
8	10	Out	50	25	Aspirate standard or sample solution
9	8	Out	70	20	Aspirate R-B solution
10	10	Out	50	25	Aspirate standard or sample solution
11	9	Out	50	40	Aspirate iron(III)
12	8	Out	70	20	Aspirate R-B solution
13	1	Out	70	1800	Dispense all solution through detector to waste

3.5.6.1.1 The effect of sequence order

The effect of sequence order for determination of ascorbic acid were designed 3 segment presented in Table 20. The sequence order was modified according to segment profile for an analysis of iron(III) content which fixed total volume at 300 µL.

Table 20 The different sequence profiles for determination of ascorbic acid

Sequence No.	Sequence order	Volume (µL)
1	R-B/Iron(III)/SD/R-B/SD/Iron(III)/SD/ R-B/SD/Iron(III)/R-B	20/40/25/20/25/40/25/ 20/25/40/20
2	R-B/SD/Iron(III)/R-B/SD/Iron(III)/SD/ R-B/SD/Iron(III)/R-B	20/25/40/20/25/40/25/ 20/25/40/20
3	R-B/SD/Iron(III)/SD/R-B/SD/Iron(III)/SD/ R-B/SD/Iron(III)/SD/R-B	20/10/40/20/20/20/40/20/ 20/20/40/10/20

3.5.6.1.2 The effect of concentration of iron(III)

The effect of iron(III) concentration on the absorption signal of ascorbic acid determination was studied in the range 10-50 mg L^{-1}.

3.5.6.1.3 The effect of aspirate volume of iron(III) solution

The effect of aspirate volume of iron(III) solution on the absorption signal of ascorbic acid determination was studied in the range 60-180 µL**.**

3.5.6.1.4 The effect of aspirate volume of R-B solution

The effect of aspirate volume of R-B solution on the absorption signal of ascorbic acid determination was studied in the range 60-120 µL**.**

3.5.6.1.5 The effect of aspirate volume of ascorbic acid standard/ sample solution

The effect of aspirate volume of ascorbic acid standard/ sample solution on the absorption signal of ascorbic acid determination was studied in the range 60-120 µL**.**

3.5.6.1.6 The effect of mixing coil length

The effect of mixing coil length on the absorption signal of ascorbic acid determination was studied at the mixing coil in the range 0-150 cm.

3.5.6.1.7 The effect of dispensing flow rate

The effect of dispensing flow rate on the absorption signal of ascorbic acid determination was studied at the dispensed flow rate of 30 – 110 µL s^{-1}.

3.6 Method Validation

3.6.1 Method validation for determination of iron(III)

3.6.1.1 Linearity

The linear calibration graph for determination of iron(III) in sample was performed by measurement the absorbance of iron standard concentration in the range of 0.2-10 mg L^{-1} under the optimum conditions.

3.6.1.2 Precision

The precision of the proposed method were studied as repeatability. The repeatability was determined by analyzing 10 time replicates of 0.3, 3, 5 and 7 mg L^{-1} of iron(III) standard and calculated as the relative standard deviation using equation 1.

$$\% \text{ RSD} = \frac{SD}{\bar{X}} \times 100 \qquad \text{..... 1}$$

When % RSD = Percentage relative standard

SD = Standard deviation

$\bar{X}$ = Average

3.6.1.3 Accuracy

The accuracy of the proposed method was determine by addition of iron(III) standard solution into samples various concentrations of 3, 5, 7 mg L^{-1} for water samples and 2, 4 mg L^{-1} for rice and vegetable samples. And then the absorbance signal was measured under the optimum experimental conditions.

The iron(III) concentration was calculated against calibration graph. Then, the percentage recovery was calculated using the equation 2.

$$\% \text{ Recovery} = \frac{(C_S - C_u)}{C_A} \times 100 \qquad \text{……..} \quad 2$$

When C_s = Concentration of fortified samples

C_u = Concentration of unfortified samples

C_A = Concentration of analyte added to the test sample

3.6.1.4 Limit of detection (LOD) and Limit of quantitation (LOQ)

The limit of detection and limit of quantitation were analyzed as minimum concentration which can be detected and calculated for both using equation 3 and 4

$$\text{LOD} = \bar{X} + \frac{3SD}{S} \qquad \text{……..} \quad 3$$

$$\text{LOQ} = \bar{X} + \frac{10SD}{S} \qquad \text{……..……..} \quad 4$$

When S = Slope of calibration curve

SD_{blank} = Standard deviation of concentration of iron(III) in blank

3.6.2 Method validation for determination of ascorbic acid

3.6.2.1 Linearity

The linear calibration graph for determination of ascorbic acid was performed by measurement the absorbance of ascorbic acid standard concentration in the range 4-50 mg L^{-1} under the optimum conditions.

3.6.2.2 Precision

The precision of the proposed method was studied as repeatability. The repeatability was determined by analyzing 10 time replicates of 10 and 20 mg $L^{-1}$of ascorbic acid standard and calculated as the relative standard deviation using equation 1.

3.6.2.3 Limit of detection (LOD) and Limit of quantitation (LOQ)

The limit of detection and limit of quantitation was analyzed as minimum concentration which can be detected and calculated for both using equation 3 and 4.

3.6.2.4 Label amount

Ascorbic acid content in pharmaceutical samples obtained by proposed method was compared with the amount ascorbic acid on the label using the equation 5.

$$\%\text{Label} = \frac{[\text{As}]_A}{[\text{As}]_L} \times 100 \qquad \text{……..} \quad 5$$

When $[\text{As}]_A$ = Concentration of ascorbic acid obtained by the developed method

$[\text{As}]_L$ = Concentration of ascorbic acid on pharmaceutical label

3.7 Sample preparation

3.7.1 Collection and preparation of water, rice and local vegetable samples

Water samples were tap water and drinking water which were collected from Kantharawichai District, Mahasarakham Province, Thailand in 1 L of polyethylene bottle. The samples were filtered and acidified with 2 mL of concentrated HNO_3. Next, H_2O_2 0.5 mL was also added into sample to prevent metal precipitation. Then, samples were kept at 4 °C in refrigerator before analysis.

Rice samples were purchased from online shopping and supermarket at Maung Roi-et District, Roi-Et Province, Thailand. The rice samples was dried in an oven for 4 h at 60 °C. Rice sample was homogenized and weighed one gram into crucible for digestion with 1 mL of HNO_3 until dry ash in the furnace at 450°C for 16 h. After that, dry ash sample was then treated with 1 mL of concentrated HNO_3 and was ashed again for 6 h at 450 °C. Then, the residue ash was dissolved in 1 mL of concentrated HNO_3 and filtered through filter paper (Pan et al. 2013). The sample was diluted to 10 mL with distilled water.

Local vegetable samples were collected from Srisomdej and Maung Roi-et District, Roi-Et Province, Thailand. An samples were dried in an oven at 60 °C for

24 h. After that, sample was homogeneously mixed. Then one gram of vegetables was prepared using the same procedures as well as rice samples that described previously.

3.7.2 Collection and preparation of pharmaceutical samples

Tablets containing ascorbic acid were obtained from subdistrict health promotion hospital in Roi-Et Province, Thailand. The sample were selected and bought direct from local drug stores in Roi-Et Province, Thailand. Ten vitamin C tablets were weighed and pulverized to fine powder, then weighed amount 0.1-0.2 g and dissolved in deionized water. After that sample solution was filtered through Whatman No.1 and adjusted into 50 mL of volumetric flask. An aliquot of the solution was filtered through a 0.45 µm nylon filter and dilution by deionized water before analysis by sequential injection analysis.

3.8 Interference effect

3.8.1 The effect of various interferences to iron determination

The probable disturbing ions were studied in water rice and vegetable samples as Na^{+}, SO_4^{2-} ,Ni^{2+}, Fe^{2+} ,Ca^{2+} ,CH_3COO^{-} , Al^{3+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, Mn^{2+}, Br^{-} ,NO_2^{-}, Cl^{-}, CO_3^{2-}, and NO_3^{-}. Various concentrations of species ion were spiked into a standard solution of 0.5 mg L^{-1} iron(III). The tolerance is defined as the interference species concentration causing an error smaller/higher than ±5% for determination of the analyte of interest (Pragourpun et al. 2015). The prepared solution was then taken to measure the absorbance at 565 nm and calculated using equation 6

$$\% \text{ difference} = \frac{(\text{Abs. Iron(III)}) - (\text{Abs.Iron(III)} + \text{Int.})}{(\text{Abs. Iron(III)})} \times 100 \quad \ldots\ldots\ldots\ldots\ldots 6$$

When Abs. Iron(III) = Absorbance of iron(III)
Abs. Iron(III) + Int. = Absorbance of iron(III) + interference

3.8.2 The effect of interference to ascorbic acid assay

The effects of various interferences possibly present in pharmaceutical samples such as critic acid was investigated. Various concentrations of critic acid were spiked into a standard solution of 15 mg L^{-1} ascorbic acid. The prepared solution was then taken to measure the absorbance at 565 nm and calculated using equation 6.

3.9 Standard method

3.9.1 Determination of vitamin C concentration by titration

The redox titration method was utilized as standard reference to determine ascorbic acid in order to compare the results obtained by the developed method. The titration method was briefed. Accurate 25 mL of sample solution from 3.7.2 was transferred in conical flask 250 mL. Then, 10 mL of KIO_3 (0050 mol L^{-1}), 5 mL of KI (0.5 mol L^{-1}), 5 mL of H_2SO_4 (0.15 mol L^{-1}) and 1 mL of starch indicator solution were added. Each of the sample solutions was then titrated against $Na_2S_2O_3$ (0.030 mol L^{-1}) until the solution turns blue to colorless, which indicate that the end point of the reaction. The titration was repeated three times for each of the pharmaceutical samples. The titration reaction was presented at equation 7-9.

$$IO_3^-(aq) + 8I^-(aq) + 6H^+(aq) \longrightarrow 3I_3^-(aq) + 3H_2O(l) \quad \text{..... 7}$$

$$C_6H_8O_6(aq) + I_3^-(aq) \longrightarrow C_6H_6O_6(aq) + 3I^-(aq) + 2H^+(aq) \quad \text{..... 8}$$

$$I_3^-(aq) + 2S_2O_3^{2-}(aq) \longrightarrow 3I^-(aq) + S_4O_6^{2-}(aq) \quad \text{..... 9}$$

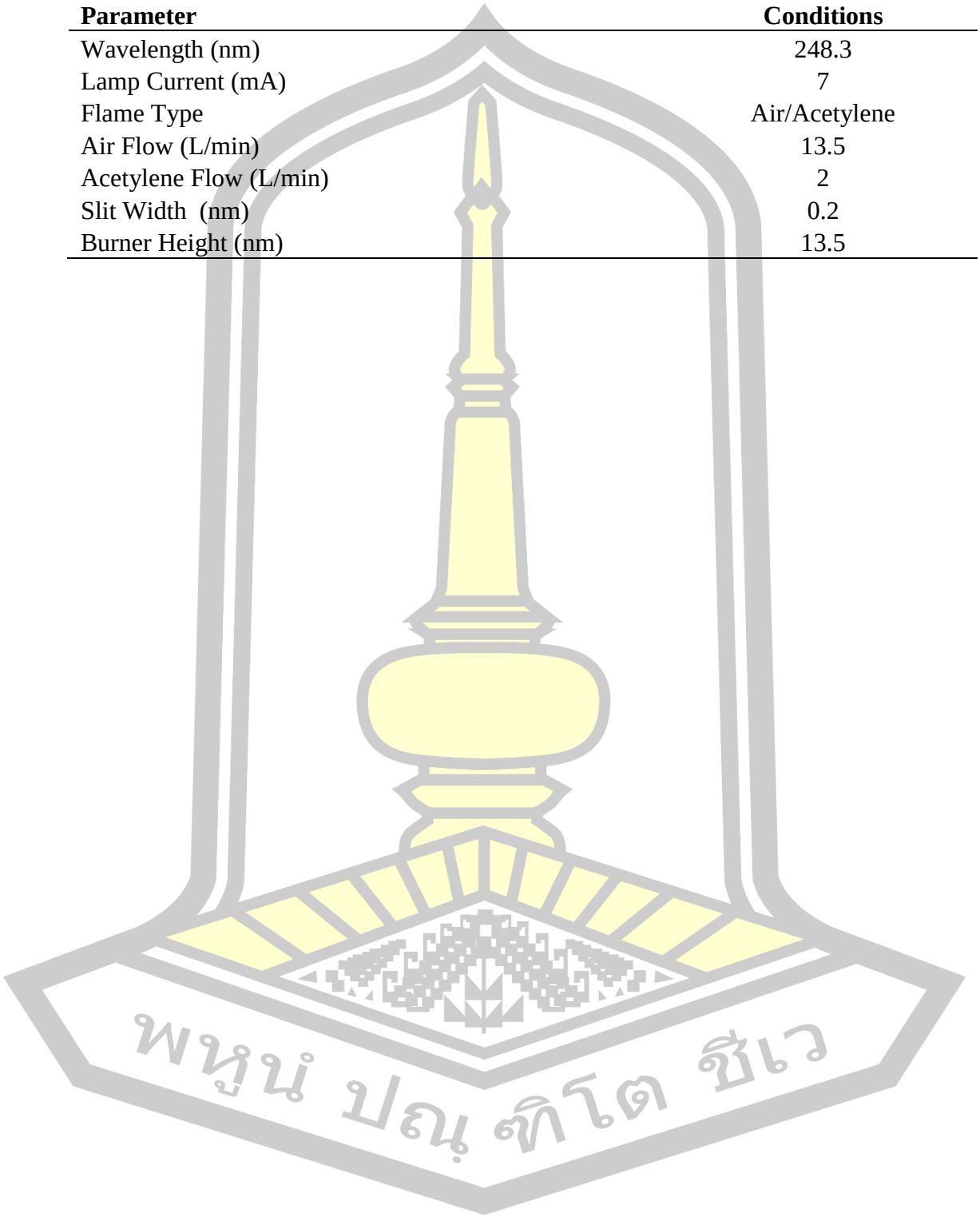
3.9.2 Determination of iron(III) concentration by flame atomic absorption spectrometry (FAAS)

Flame atomic absorption spectrometry was used as standard method for the quantification of iron(III) in real samples. Moreover, the results obtained by FAAS were utilized to compare with the results achieved from the proposed method. A calibration curve for the determination of iron in water, rice and vegetable samples by FAAS in the concentration range 0.2-10.0 mg L^{-1} iron(III) was established using the following procedure. An appropriate amount of working standard solution of iron was transferred in 25 mL volumetric flask and then was the volume adjusted with deionized water.

For the determination of iron(III) in water rice and vegetable samples, the 1 mL sample volume from 3.7.1 was transferred into 25 mL volumetric flask. After that, the volume was adjusted with deionized water. The FAAS was tested using experimental conditions as shown in Table 21.

Table 21 Condition of the FAAS instrument for iron content analysis

Parameter	**Conditions**
Wavelength (nm)	248.3
Lamp Current (mA)	7
Flame Type	Air/Acetylene
Air Flow (L/min)	13.5
Acetylene Flow (L/min)	2
Slit Width (nm)	0.2
Burner Height (nm)	13.5



CHAPTER IV

RESULTS AND DISCUSSION

4.1 Preliminary study on the use of betel nut extracts in metal analysis

The important chemicals found in betel nut were phenolic compounds as tannins. These compounds can form complexes with iron(III) metal.

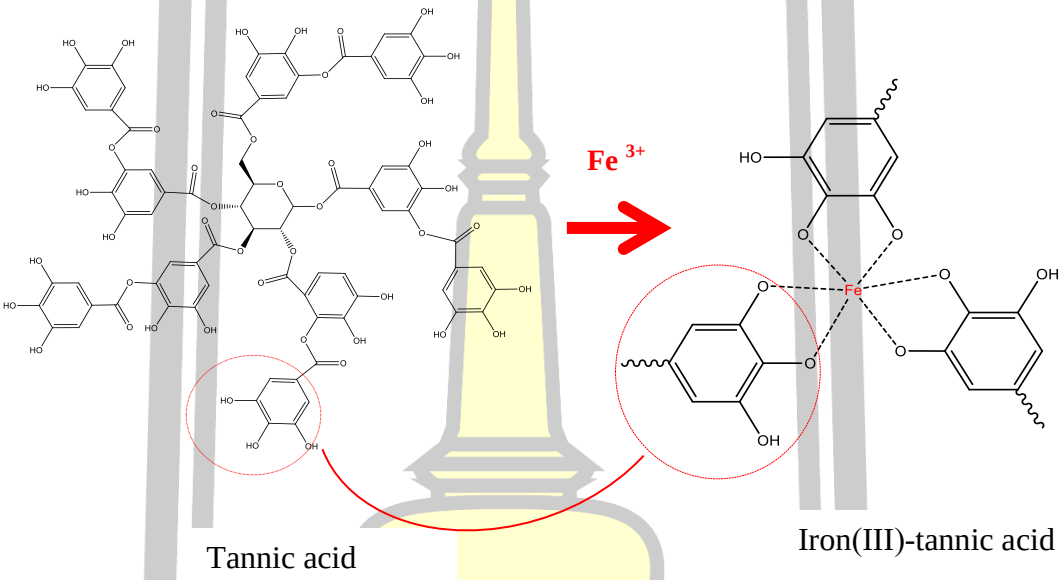


Figure 8 The structure of the complex between tannic acid and iron(III)- tannic acid

The hydroxyl groups in tannic acid can react with the iron(III) for the formation of metal complexes (Çakar et al.2016). Complexes are formed between iron(III) and phenolic groups with the presence of a third adjacent hydroxyl (pyrogallols) increase the stability of the complexes. The possible mechanism of complexation is shown in Figure 8.

4.1.1 Preliminary investigation of selectivity of the natural reagent with some metal

The complexation of the betel nut extract solution and some other metal ions was preliminarily investigated. The ability to form complexes depends on the experimental conditions (pH of the solution) and stability of complexes. Various ions such as iron(III), iron(II), lead(II), zinc(II), cadmium(II), copper(II), antimony(III), calcium(II), bismuth(III), manganese(II) and aluminum(III) were tested to react with the extracted solution at pH 5.5. The results are presented in Figure 9(a). It was observed that only iron(III) can associated with natural reagent resulting to dark-purple color, which was provided a maximum wavelength at 565 nm shown in Figure

9(b), while for the other metal ions no color change was observed. Therefore, the determination of iron(III) using natural reagent extracted from betel nut was selected since the other metal ions did not interfere with iron(III) detection.

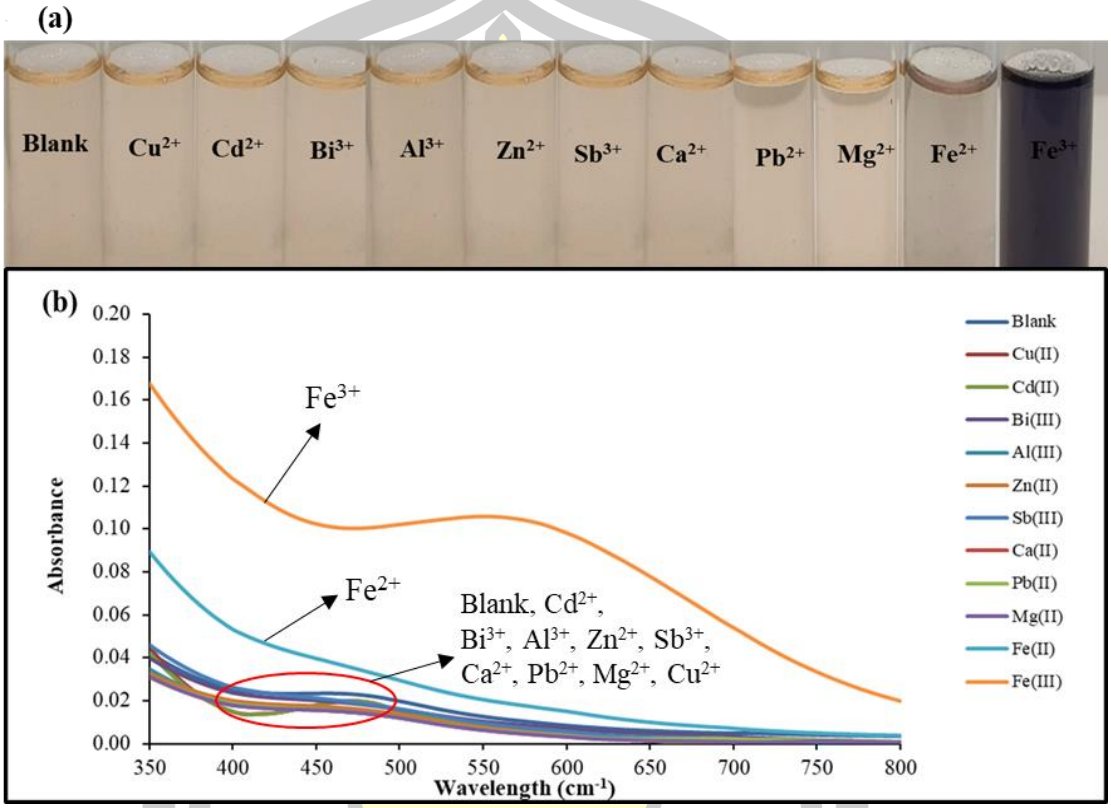


Figure 9 (a) digital images of color changes observed in addition of difference metals to the betel nut solution in acetate buffer pH 5.5 at room temperature and **(b)** UV-Vis spectra of betel nut extract solution contained different metals at a concentration of 10 mg L^{-1} in acetate buffer pH 5.5

4.2 Characterization of the complex formation

Tannic acid is a major phenolic compounds found in betel nut (Rathod et al. 2015). It may be acted as metal chelator to associate with iron(III) in this study. There are several reports was investigated about the tannic acid with iron(III) (Çakar et al. 2016). However, to prove this hypothesis, FT-IR and UV-Vis spectroscopy were utilized to characterize of complex formation between iron(III)-tannic acid, and iron(III)- betel nut complexes. The complexes were synthesized by adding of 100 mg L^{-1} iron(III) standard solution into 0.5 mg L^{-1} of tannic acid standard or betel nut extracted solution under the 0.7 mol L^{-1} acetate buffer pH 5.5. The purple solid of iron (III)- tannic acid/ betel nut complexes was obtained. The infrared spectra of the betel nut powder, tannic acid standard, iron(III)-betel nut complex, iron(III)-tannic acid complex were presented in Figure 10 (a), 10 (b) and 10 (c), respectively. The wavenumber of tannic acid, betel nut, iron(III)-tannic acid complex and iron(III)-betel nut complex was summarized in Table 22 and 23, respectively.

It was found to be, the FTIR spectra of the betel nut powder are shown the similar characteristic peaks as well as observed in tannic acid standard at wavenumber (Figure 10 (a) and Table 22) 3373 cm^{-1} (O-H stretch), 1700 cm^{-1} (C=O stretch), 1647 cm^{-1} (C-C stretch) ,1522 cm^{-1} (C-C stretch), 1420 cm^{-1} (C-O stretch), 1373 cm^{-1} (C-O stretch), 1245 cm^{-1} (O-H in-plane deformation), 1086 cm^{-1} (C-O stretch) and 831 cm^{-1} (C-H out-of-plane bending), respectively. Therefore, a tannic acid is the major phenolic compound in extracts powder. The wavenumber obtained from the reaction between betel nut extract solution with iron(III) was similarly achieved by the reaction between tannic acid and iron(III) as presented in Table 23. Furthermore, the characteristic peak of the carbonyl group (1700 cm^{-1}) and the characteristic peak of the C-O bond stretching vibration of phenolic changed after reaction between betel nut and iron(III) under pH 5.5, in which the band was shifted from 1377.17 cm^{-1} (spectrum of betel nut) to 1384.43 cm^{-1} (spectrum of the iron(III)-betel nut complex) and the band was shifted from 1345.07 cm^{-1} (spectrum of tannin acid) to 1406.19 cm^{-1} (spectrum of the iron(III)-tannic acid complex) (Li et al. 2016). Moreover, the peaks that occur between 1,000 and 1,300 cm^{-1} were very broad in the both iron(III)-betel nut and iron(III)-tannic acid complex due to the band spectrum were shifted (Figure 10 (b) and 10 (c)). So, it clearly shown that the C-O and O-H of phenolic in the betel nut can be reacted with the iron(III) resulting to metal complex formation.

Moreover, the absorption spectra of the iron(III)- betel nut complexes and iron(III)-tannic acid were measured by spectrophotometry as demonstrated the spectra in Figure 11. This maximum absorption wavelength and the color product obtained between the complex of iron(III)-betel nut was resembled with the iron(III)-tannic acid complex. Hence, we can confirm that betel nut crude extracted contain of tannic acid.

Table 22 Summarized FTIR spectra of standard tannic acid and betel nut powder

Wavenumber (cm^{-1})		Assignment functional groups
Tannic acid	**Betel nut**	
3320.16	3373.85	(–OH) stretch of H-bonded
1700.76	1700.66	(C=O) stretch of benzene ring
1620.23	1647.8	(C-C) stretch and (C-H) deformation in plant of benzene ring
1539.69	1522.28	(C-C) stretch and (C-H) deformation in plane of benzene ring (C-O) stretch of phenolic
1429.41	1420.7	(C-C) stretch of benzene ring and methylene, (C-O) stretch of phenolic
1345.07	1373.17	(C-O) stretch and (O-H) deformation of phenolic, (C-C) stretch and deformation in plane of benzene ring
1205.94	1245.85	(O-H) deformation in plane of phenolic and carboxylic acid, (C-C) stretch and (C-H) deformation in plane of benzene ring
1027.46	1086.96	(C-O) stretch of phenolic, ether and carboxylic acid
759.74	831.77	(C-H) deformation out of plane of benzene ring

Table 23 Summarized FTIR spectra of iron(III)-tannic acid and iron(III)-betel nut complexs

Wavenumber (cm^{-1})		Assignment functional groups
Iron(III)-tannic acid	**Iron(III)- betel nut**	
3436.25	3431.17	(–OH) stretch of H-bonded
1686.25	1686.5	(C=O) stretch of benzene ring
1634.74	1641.27	(C-C)stretch and (C-H) deformation in plant of benzene ring
1560.73	1549.85	(C-C) stretch and (C-H) deformation in plane of benzene ring (C-O) stretch of phenolic
1526.63	1515.75	(C-C)stretch of benzene ring and methylene, (C-O) stretch of phenolic
1417.8	1418.53	(C-O)stretch and (O-H) deformation of phenolic, (C-C) stretch and deformation in plane of benzene ring
1406.19	1384.43	(O-H) deformation in plane of phenolic and carboxylic acid, (C-C) stretch and (C-H) deformation in plane of benzene ring
822.86	823.51	(C-H) deformation out of plane of benzene ring
662.52	663.97	(C-H) torsion of benzene ring

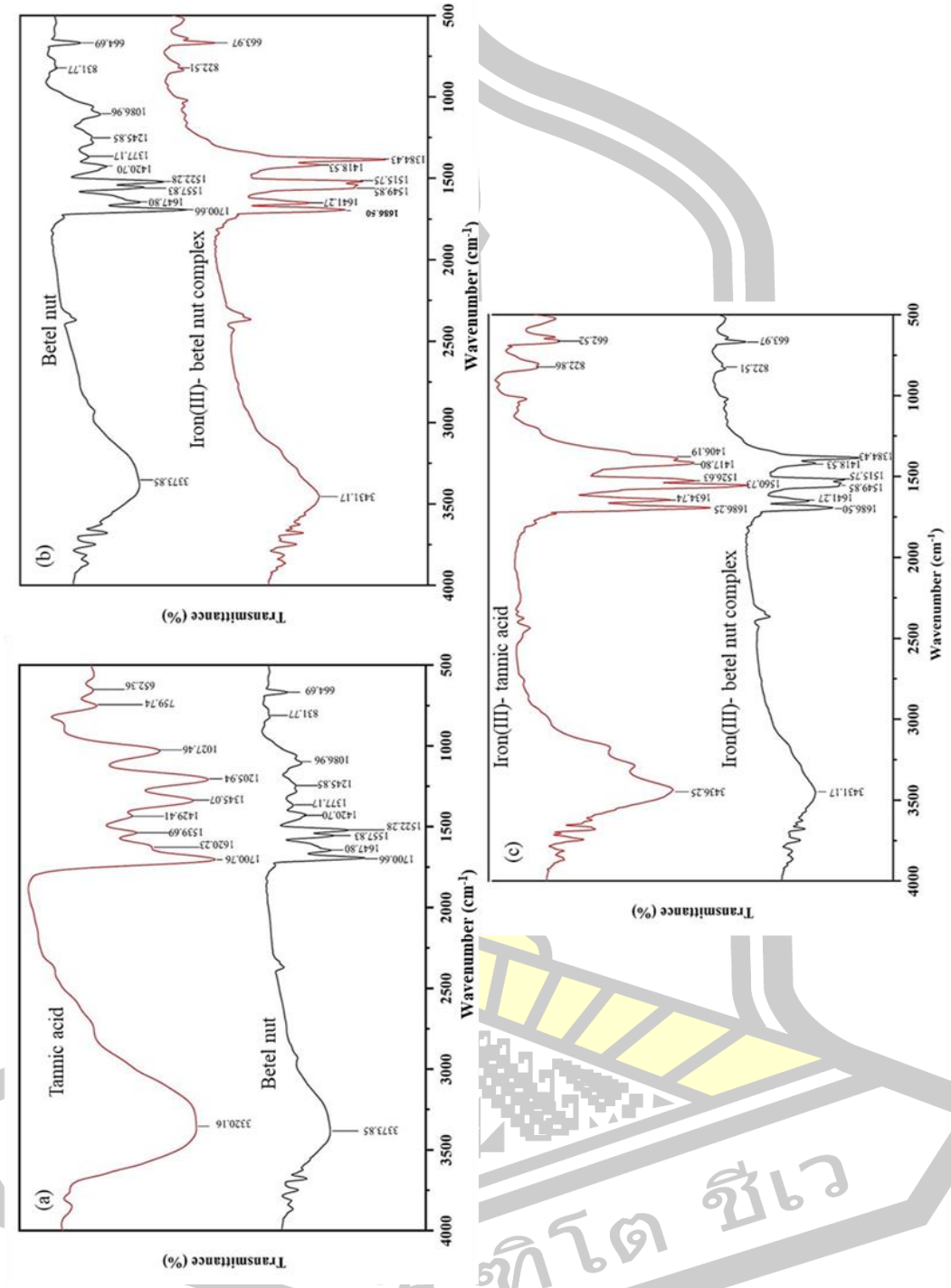


Figure 10 FTIR spectra of **(a)** tannic acid and betel nut; **(b)** iron(III)-tannic acid and iron(III)-betel nut ; **(c)** betel nut and iron(III)-betel nut complex

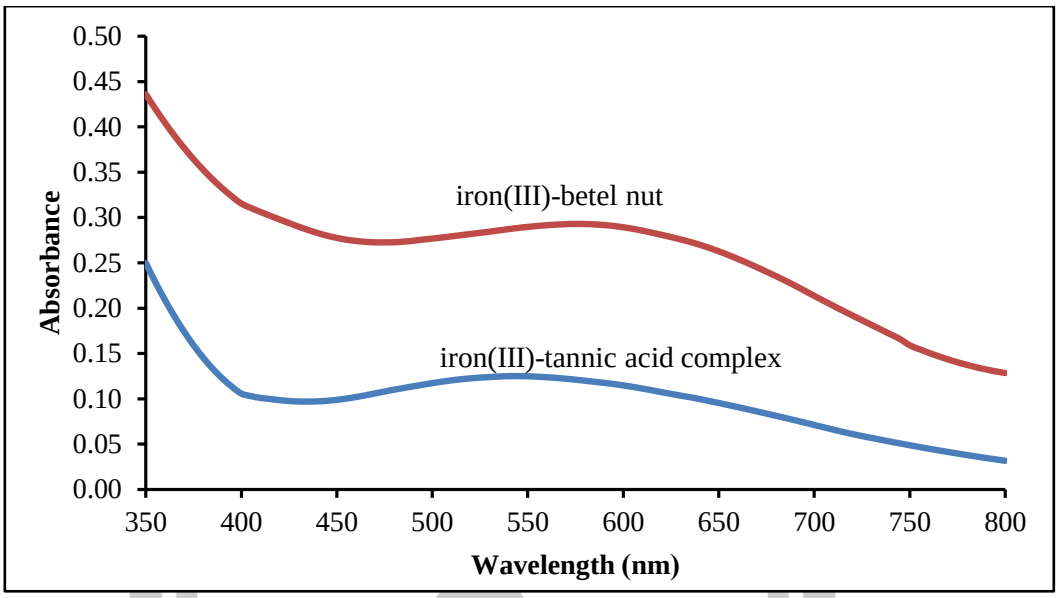


Figure 11 Absorption spectra of the complex formed between iron(III)- betel nut extract solution and the standard solution of tannic acid – iron(III) at pH5.5

4.3 Optimization of natural reagent extraction

4.3.1 Type of solvent for extraction of natural reagent

In general, choosing the extract solvent is the important parameter optimization. In this study, natural reagent solution was obtained by stirring with many type of solvent as deionized water with and without heat, and organic solvent without heat as methanol, acetone, acetonitrile, ethanol and 50% ethanol for 20 min. Absorption signal of complex using different type solvent at 565 nm were recorded. The results were shown in Table 24 and Figure 12.

Table 24 The effect of difference solvent extraction on the absorbance of iron(III) complex

Difference solvent extraction	Absorbance		Net signal
	Blank	Iron(III) complex	
Acetone	0.1257	0.1826	0.0571
	0.1254	0.1827	
	0.1256	0.1826	
$\bar{X}$ ±SD	0.1256±0.0002	0.1826±0.0001	
Acetonitrile	0.0253	0.0899	0.0645
	0.0252	0.0898	
	0.0251	0.0894	
$\bar{X}$ ±SD	0.0252±0.0001	0.0897±0.0003	
Deionized water	0.0388	0.1277	0.0889
	0.0389	0.1276	
	0.0387	0.1277	
$\bar{X}$ ±SD	0.0388±0.0001	0.1277±0.001	
50% v/v ethanol	0.0725	0.1579	0.0852
	0.0727	0.1578	
	0.0729	0.1579	
$\bar{X}$ ±SD	0.0727±0.0002	0.1579±0.0001	
Ethanol	0.0770	0.1424	0.0651
	0.0790	0.1423	
	0.0760	0.1425	
$\bar{X}$ ±SD	0.0773±0.0015	0.1424±0.0001	
Hot water	0.0685	0.1699	0.1013
	0.0685	0.1698	
	0.0686	0.1699	
$\bar{X}$ ±SD	0.0685±0.0001	0.1699±0.0001	
Methanol	0.1564	0.2078	0.0515
	0.1560	0.2077	
	0.1562	0.2076	
$\bar{X}$ ±SD	0.1562±0.0002	0.2077±0.0001	

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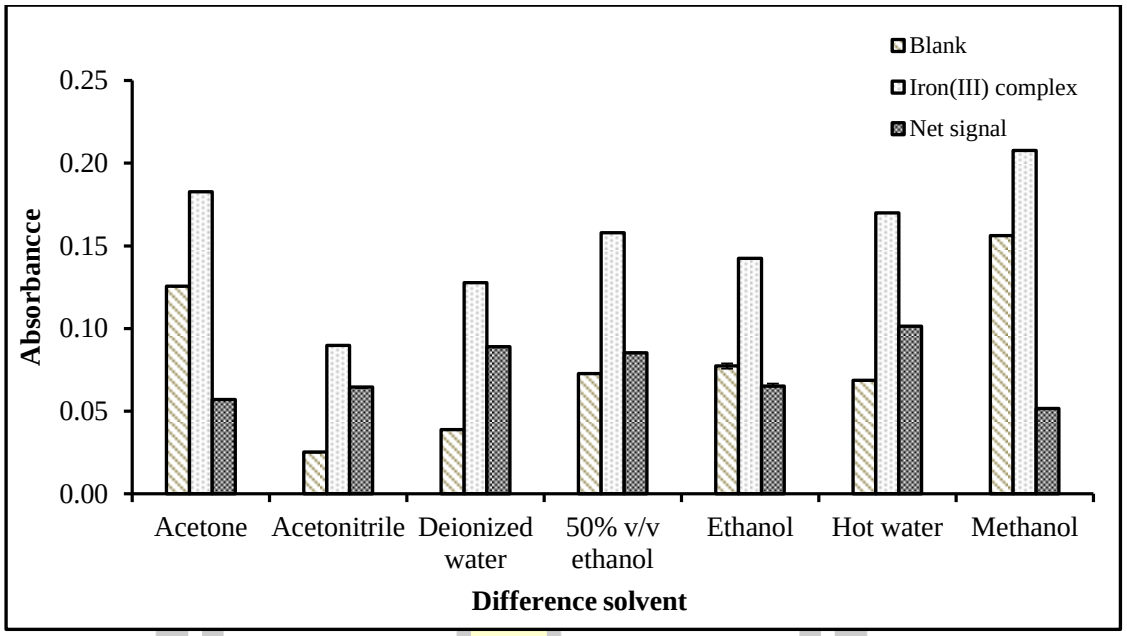

Figure 12 Effect of iron(III)-betel nut complex with different extraction solvents

The result showed that the natural reagent solution extracted from deionized water with heat (hot water) was provided highest absorbance**.** Therefore, the extraction of natural reagent was operated using water with heat which provided importance to the work as water is green solvent, compatible, inexpensive, and easily available.

4.3.2 Effect of extraction time

The extraction time was investigated from 5-60 minutes. Using a longer extraction time can be assisted to extract more phenolic compound. However, the results shown that the absorbance value was increased until the extraction time up to 20 min. After that, there is no significant difference of absorbance as seen Table 25 and Figure 13. So, extraction time of 20 min was selected for the proposed method.

Table 25 The effect of extraction time on the absorbance of iron(III) complex

Time(min)	Absorbance		Net signal
	Blank	Iron(III) complex	
10	0.0070	0.1070	0.0997
	0.0070	0.1070	
	0.0070	0.1060	
$\bar{X}$ ±SD	0.0070±0.0000	0.1067±0.0006	
20	0.0080	0.1180	0.1107
	0.0070	0.1180	
	0.0080	0.1190	
$\bar{X}$ ±SD	0.0077±0.0006	0.1183±0.0006	
30	0.0090	0.1200	0.1047
	0.0090	0.1110	
	0.0090	0.1100	
$\bar{X}$ ±SD	0.0090±0.0000	0.1137±0.0055	
40	0.0100	0.1140	0.1040
	0.0100	0.1130	
	0.0090	0.1140	
$\bar{X}$ ±SD	0.0097±0.0006	0.1137±0.0006	
60	0.0110	0.1160	0.1037
	0.0110	0.1140	
	0.0120	0.1150	
$\bar{X}$ ±SD	0.0113±0.0006	0.1150±0.0010	

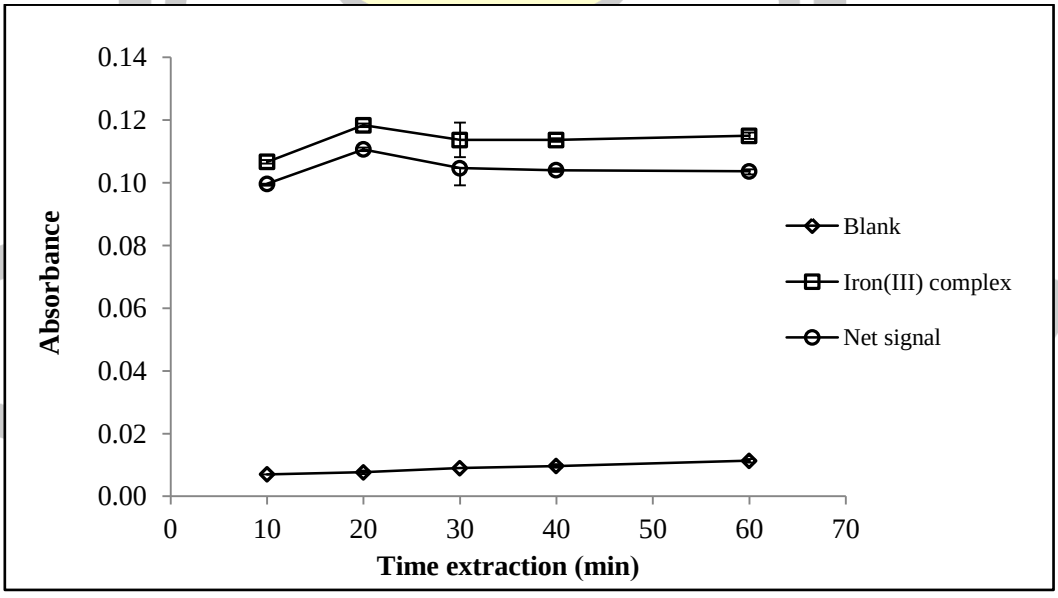

Figure 13 The effect of extraction time on the sensitivity for iron(III) determination

4.3.3 Effect of natural reagent mass

The effect of betel nut mass on the sensitivity of the determination iron(III) was studied by changing the weight of betel nut powder from 0.1-5.0 g. Results was demonstrated in Table 26 and Figure 14.

Table 26 The effect of on mass of betel nut the absorbance of iron(III) complex

Mass of betel nut (g)	Absorbance		Net signal
	Blank	Iron(III) complex	
0.1	0.0100	0.0900	0.0810
	0.0110	0.0910	
	0.0100	0.0930	
$\bar{X}$ ±SD	0.0103±0.0006	0.0913±0.0015	
0.3	0.0180	0.1190	0.0997
	0.0180	0.1160	
	0.0170	0.1170	
$\bar{X}$ ±SD	0.0177±0.0006	0.1173±0.00153	
0.5	0.0350	0.1480	0.1127
	0.0350	0.1480	
	0.0350	0.1470	
$\bar{X}$ ±SD	0.0350±0.0000	0.1477±0.0006	
1	0.0390	0.1360	0.0970
	0.0420	0.1380	
	0.0410	0.1390	
$\bar{X}$ ±SD	0.0407±0.0015	0.1377±0.0015	
2	0.0640	0.1620	0.0980
	0.0640	0.1610	
	0.0640	0.1630	
$\bar{X}$ ±SD	0.0640±0.0000	0.1620±0.0010	
3	0.1110	0.2090	0.0983
	0.1100	0.2090	
	0.1110	0.2090	
$\bar{X}$ ±SD	0.1107±0.0006	0.2090±0.0000	
4	0.1130	0.2080	0.0947
	0.1140	0.2090	
	0.1130	0.2070	
$\bar{X}$ ±SD	0.1133±0.0006	0.2080±0.0010	
5	0.1150	0.2100	0.0940
	0.1150	0.2090	
	0.1170	0.2100	
$\bar{X}$ ±SD	0.1157±0.0012	0.2097±0.0006	

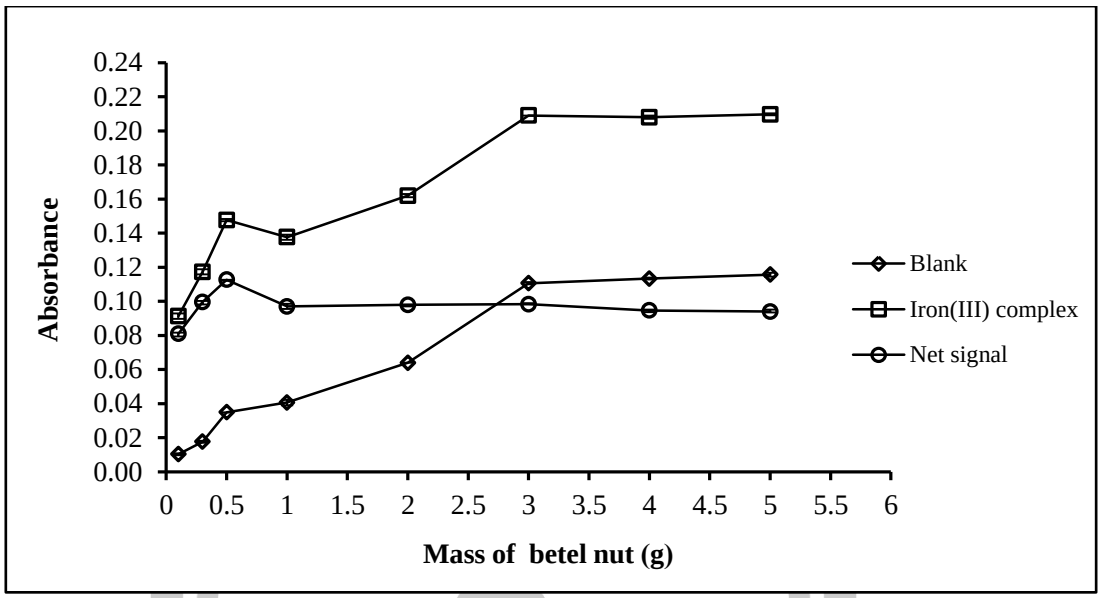

Figure 14 The effect of on mass of betel nut the sensitivity for iron(III) determination

It was found that absorbance signal was increased with increasing a mass of betel nut powder from 0.1 to 0.5 g. The net absorption signal decreases until constant for reagent mass more than 1.0 g because the blank absorbance was increased but betel nut-iron complex signal was not significant increased resulting to decrease of net absorbance value. Hence, 0.5 g of betel nut was chosen for the developed method in order to provide the best cost effective and precision of the method.

4.4 Optimum conditions for the quantification of iron(III) using natural reagent

4.4.1 Effect of pH

Obviously, the pH of the solution is one of the most important parameter for complex formation between metal ions and ligands. Therefore, the effect of pH was necessary investigated. The effect of pH on the reaction between iron(III)-betel nut solution was investigated in the range of 4.5- 10. At pH below pH 5.5, the functional group of ligands may be protonate released, thus avoiding complex formation. But pH higher than 5.5 the iron(III) might form $Fe(OH)_3$ precipitate and may not be suitable for complex formation thus low sensitivity was resulted. The absorption of each pH are shown in Table 27 and Figure 15.

Table 27 The effect of buffer solution of betel nut the absorbance of iron(III) complex

Different pH	Absorbance		
	Blank	**Iron(III) complex**	**Net signal**
4.5	0.0515	0.1123	0.0605
	0.0516	0.11	
	0.0514	0.1136	
$\bar{X}$ ±SD	0.0515±0.0004	0.1120±0.0018	
5	0.053	0.1408	0.0866
	0.055	0.1405	
	0.054	0.1406	
$\bar{X}$ ±SD	0.054±0.0010	0.1406±0.0002	
5.5	0.0436	0.1492	0.1056
	0.0436	0.1492	
	0.0436	0.1492	
$\bar{X}$ ±SD	0.0436±0.0000	0.1492±0.0000	
6	0.0600	0.1050	0.0450
	0.0599	0.1049	
	0.0600	0.1050	
$\bar{X}$ ±SD	0.0600±0.00005	0.1049±0.00005	
6.5	0.0760	0.1069	0.0313
	0.0760	0.1070	
	0.07560	0.1068	
$\bar{X}$ ±SD	0.0756±0.0006	0.1069±0.0001	
7	0.0833	0.1076	0.0244
	0.0831	0.1075	
	0.0832	0.1076	
$\bar{X}$ ±SD	0.0832±0.0001	0.1076±0.00005	
8	0.0920	0.1153	0.0229
	0.0916	0.1151	
	0.0934	0.1153	
$\bar{X}$ ±SD	0.0923±0.0001	0.1152±0.0001	
9	0.1007	0.1100	0.0112
	0.1009	0.1140	
	0.1008	0.1120	
$\bar{X}$ ±SD	0.1008±0.0001	0.1120±0.0020	
10	0.1196	0.1330	0.0124
	0.1196	0.1310	
	0.1196	0.1320	
$\bar{X}$ ±SD	0.1196±0.0000	0..1320±0.0010	

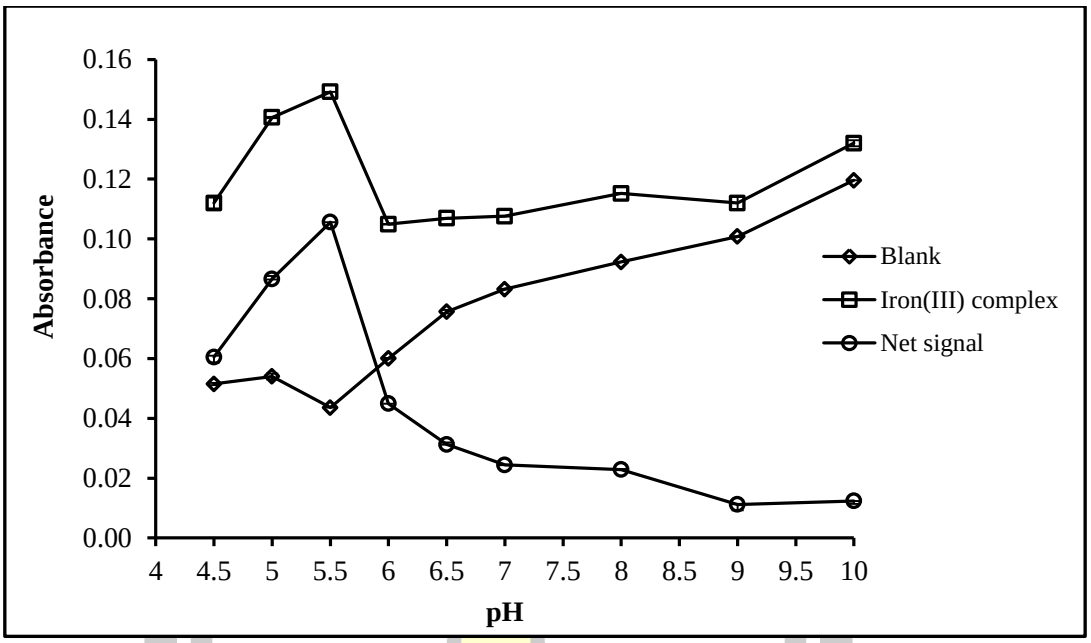

Figure 15 UV-Vis absorption spectra of the betel nut extract solution contained different buffer on complex formation of iron(III)

4.4.2 Effect of concentration of buffer

Effect of acetate buffer concentration on the absorption signal was studied in the range of 0.1-1.0 mol L^{-1}. The appropriate concentration was affected the stability of the complexation reaction. As the results were shown in Table 28 and Figure 16.

Table 28 The effect of on concentration of buffer the absorbance of iron(III) complex

Concentration of pH (mol L^{-1})	Absorbance		Net signal
	Blank	Iron(III) complex	
0.05	0.0080	0.0810	0.0720
	0.0090	0.0810	
	0.0100	0.0810	
$\bar{X}$ ±SD	0.0090±0.001	0.0810±0.0000	
0.1	0.0070	0.0800	0.0737
	0.0060	0.0800	
	0.0060	0.0800	
$\bar{X}$ ±SD	0.0063±0.0006	0.0800±0.0000	
0.3	0.0060	0.0910	0.0857
	0.0060	0.0920	
	0.0070	0.0930	
$\bar{X}$ ±SD	0.0063±0.0006	0.0920±0.0010	
0.5	0.0040	0.1030	0.0990
	0.0050	0.1040	
	0.0040	0.1030	
$\bar{X}$ ±SD	0.0043±0.0006	0.1033±0.0006	
0.7	0.0050	0.1110	0.1057
	0.0050	0.1110	
	0.0050	0.1100	
$\bar{X}$ ±SD	0.0050±0.0000	0.1107±0.0006	
1	0.0100	0.1120	0.1000
	0.0110	0.1110	
	0.0120	0.1100	
$\bar{X}$ ±SD	0.0110±0.00010	0.1110±0.0010	

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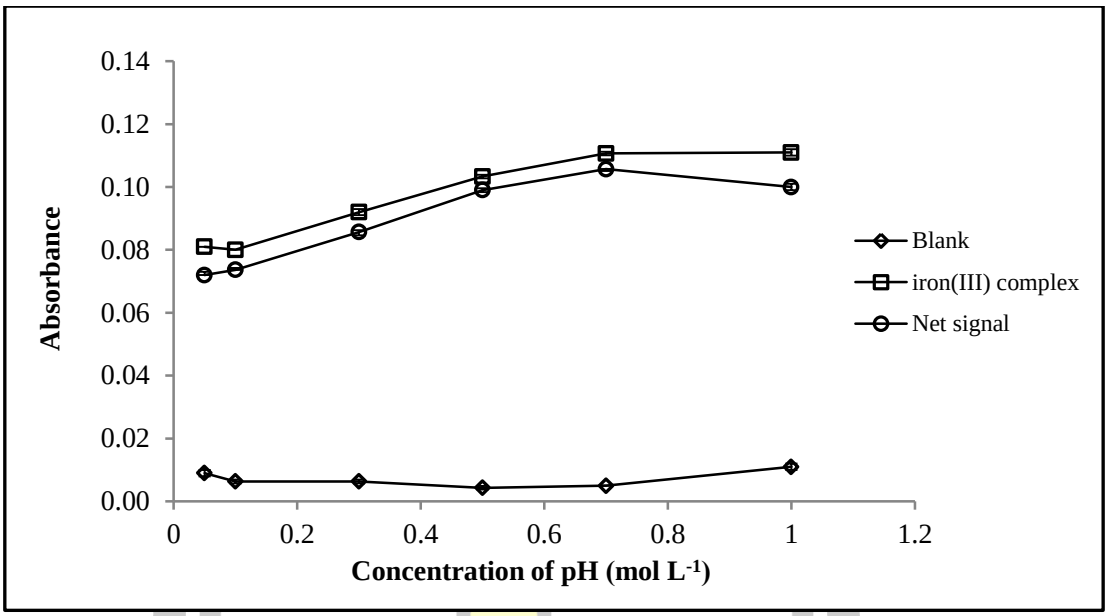

Figure 16 The effect of on concentration of buffer the sensitivity for iron(III) determination

It was found that absorbance was increased with concentration of buffer increasing up to 0.7 mol L^{-1}. After that, the absorbance was slightly decreased. So, the optimum concentration of acetate buffer selected for this experiment was 0.7 mol L^{-1} due to the high measured signal peak and high accuracy.

4.5 Optimization of SIA conditions

4.5.1 Total aspiration volume

Preliminary studied before optimized conditions of SIA system, the total aspiration volume was varied in the ranging from 50-400 µL using 10 mg L^{-1} of iron(III) mixed with 1 mL of betel nut extracted solution. The analysis signal was increased when aspiration volume increasing up to 300. Using excessive aspirated volume, the signal was stable as shown in Table 29 and Figure 17**.** Therefore, a total aspiration volume of 300 µL was selected as optimum for this purposed method.

Table 29 The effect of total volume on the absorbance of iron(III) complex

Total volume(μL)	Absorbance		Net signal
	Blank	Iron(III) complex	
50	0.0093	0.0249	0.0121
	0.0147	0.0252	
	0.0152	0.0255	
$\bar{X}$ ±SD	0.0131±0.0033	0.0252±0.0003	
100	0.0184	0.0453	0.0261
	0.0187	0.0423	
	0.0188	0.0465	
$\bar{X}$ ±SD	0.0186±0.0022	0.0447±0.002204	
150	0.0255	0.0665	0.0423
	0.0231	0.0668	
	0.0255	0.0666	
$\bar{X}$ ±SD	0.0243±0.0016	0.0666±0.0002	
200	0.0423	0.1100	0.0662
	0.0435	0.1098	
	0.0453	0.1099	
$\bar{X}$ ±SD	0.0437±0.0001	0.1099±0.0007	
250	0.0493	0.1384	0.0862
	0.0495	0.1324	
	0.0493	0.1358	
$\bar{X}$ ±SD	0.0494±0.0001	0.1355±0.0030	
300	0.0502	0.1799	0.1293
	0.0504	0.1789	
	0.0502	0.1799	
$\bar{X}$ ±SD	0.0502±0.0002	0.1795±0.0030	
350	0.0594	0.1804	0.1209
	0.0598	0.1797	
	0.0597	0.1815	
$\bar{X}$ ±SD	0.0596±0.0002	0.1805±0.0009	
400	0.0640	0.1856	0.1208
	0.0665	0.1892	
	0.0685	0.1865	
$\bar{X}$ ±SD	0.0663±0.0022	0.1871±0.0019	

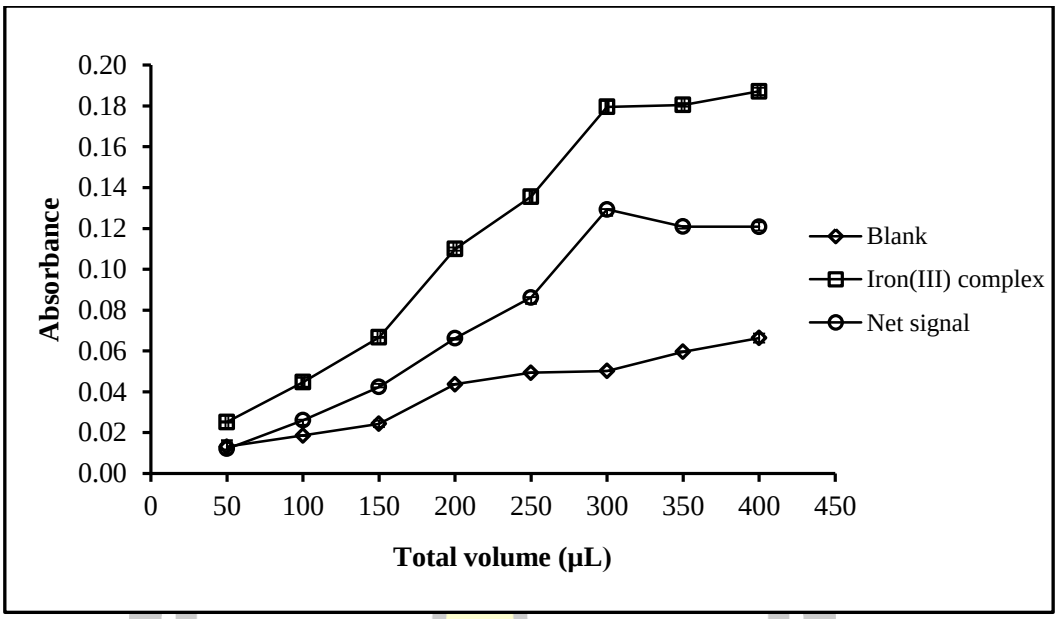

Figure 17 The effect of total volume on the sensitivity for determination of iron(III) using SIA system

4.5.1.1 Effect of aspiration sequence profile

The effect of aspiration sequence profile to aspirate the solution of standard iron(III) or sample and reagent-buffer solution pH 5.5 (R-B solution) was designed and investigated using 300 µL of total aspirated volume. Different segment profile was shown in Table 30. The results presented in Table 31 and Figure 18 found that, aspirated segment order No. 4 (R-B/SD/R-B/SD/R-B/SD/R-B) was provided the highest absorbance. So, this study, segment order No. 4 was adopted and then used in further experiments.

Table 30 Sequential profile for aspiration of iron(III) standard and reagents by the developed method

Sequence No.	Sequence order aspiration	Volume (µL)
1	R-B/SD	150/150
2	R-B/SD/R-B	75/150/75
3	R-B/SD/R-B/SD/R-B	50/75/50/75/50
4	R-B/SD/R-B/SD/R-B/SD/R-B	25/50/50/50/50/50/25
5	R-B/SD (6)	(25/25)*6

Table 31 The effect of aspiration sequence profile on the absorbance of iron(III) complex

Sequence No.	Absorbance		Net signal
	Blank	Iron(III) complex	
1	0.1399	0.2074	0.0674
	0.1371	0.2039	
	0.1388	0.2066	
$\bar{X}$ ±SD	0.1386±0.0014	0.2060±0.0018	
2	0.1367	0.2072	0.0706
	0.1364	0.2087	
	0.1369	0.2059	
$\bar{X}$ ±SD	0.1366±0.0003	0.2073±0.0014	
3	0.1280	0.2242	0.0950
	0.1280	0.2224	
	0.1284	0.2226	
$\bar{X}$ ±SD	0.1281±0.0002	0.2230±0.0010	
4	0.1218	0.2380	0.1145
	0.1208	0.2331	
	0.1201	0.2351	
$\bar{X}$ ±SD	0.1209±0.0008	0.2354±0.0024	
5	0.1256	0.2357	0.1089
	0.1258	0.2312	
	0.1259	0.2371	
$\bar{X}$ ±SD	0.1258±0.0002	0.2347±0.0031	

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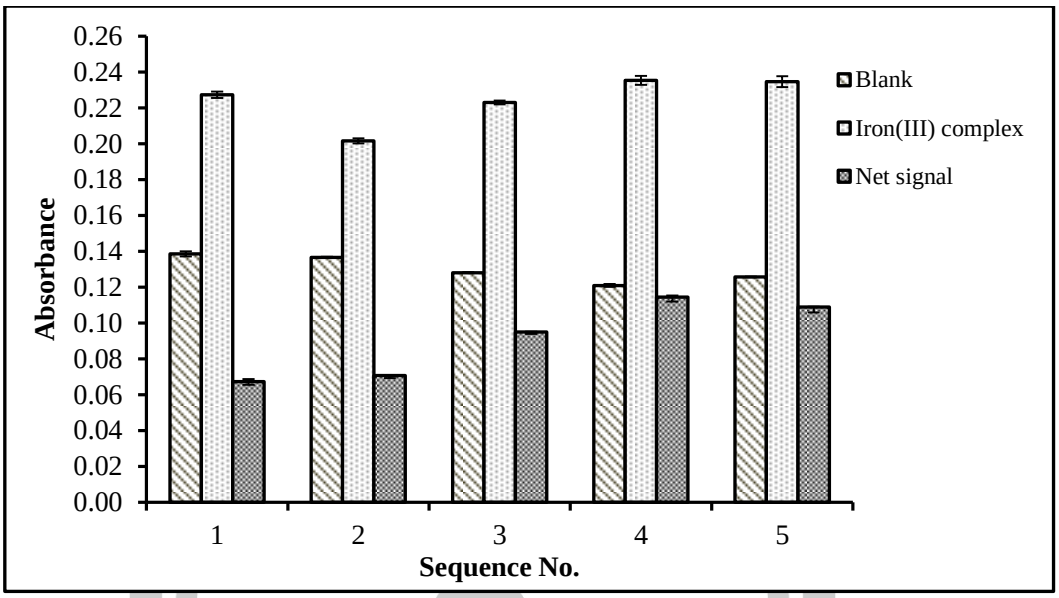


Figure 18 The effect of aspirated sequence order on the sensitivity for determination of iron(III) using SIA system

4.5.1.2 Effect of natural reagent volume dilution in buffer solution (R-B)

To simplify the reagent preparation, the offline mixing of natural reagent extracts with acetate buffers was operated before using in the SIA system. The volume of natural reagent dilution was studied from 0.5-20 mL in the total volume 25 mL. The volume of natural reagent using 7 mL in total volume 25 mL was provided highest absorbance as the result demonstrated in Table 32 and Figure 19**.** Excessive employing reagent volume the absorbance of blank was also increased. Therefore, the volume of natural reagent at 7 mL diluted to the final 25 mL was used in further experiments.

Table 32 The effect of volume of natural reagent dilution in acetate buffer (R-B) (mL) on the absorbance of iron(III) complex

Volume of reagent in acetate buffer(mL)	Absorbance		Net signal
	Blank	Iron(III) complex	
0.5	0.0306	0.0589	0.0266
	0.0322	0.0572	
	0.0333	0.0597	
$\bar{X}$ ±SD	0.0320±0.0013	0.0586±0.0013	
1	0.0364	0.0707	0.0353
	0.0369	0.0708	
	0.0356	0.0733	
$\bar{X}$ ±SD	0.0363±0.0007	0.0716±0.0015	
2	0.0381	0.0861	0.0492
	0.0386	0.0879	
	0.0380	0.0883	
$\bar{X}$ ±SD	0.0382±0.0003	0.0874±0.0012	
3	0.0404	0.1175	0.0763
	0.0418	0.1157	
	0.0408	0.1186	
$\bar{X}$ ±SD	0.0410±0.0007	0.1173±0.0015	
5	0.0421	0.1430	0.1027
	0.0431	0.1485	
	0.0442	0.1459	
$\bar{X}$ ±SD	0.0431±0.0011	0.1458±0.0028	
7	0.0472	0.1782	0.1310
	0.0471	0.1793	
	0.0490	0.1789	
$\bar{X}$ ±SD	0.0478±0.0011	0.1788±0.0006	
10	0.0532	0.1826	0.1296
	0.0533	0.1842	
	0.0554	0.1838	
$\bar{X}$ ±SD	0.0540±0.0012	0.1835±0.0008	
20	0.0637	0.1950	0.1294
	0.0660	0.1940	
	0.0642	0.1930	
$\bar{X}$ ±SD	0.0646±0.0012	0.1940±0.0010	

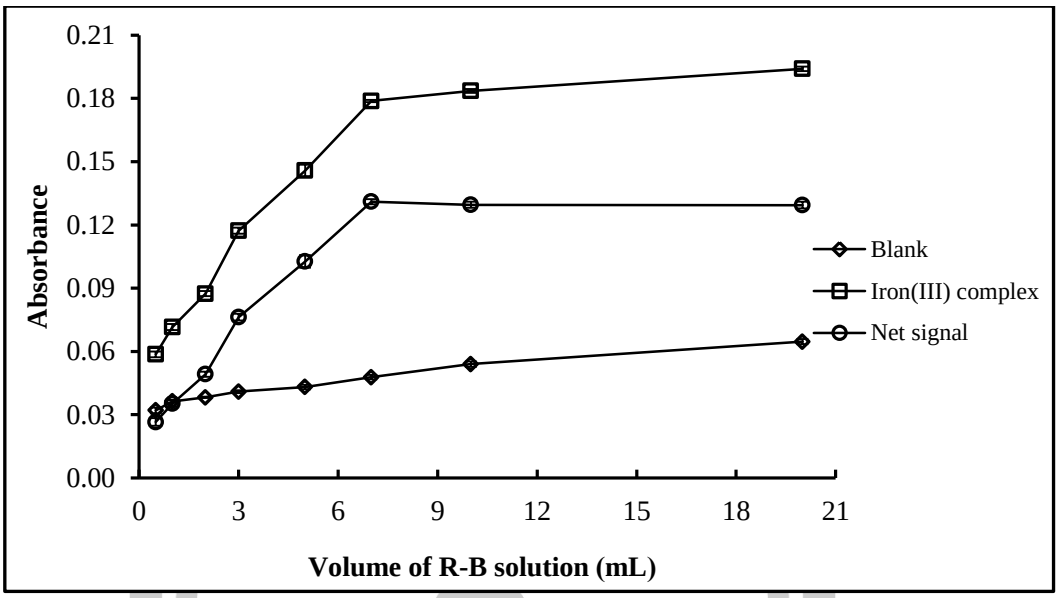


Figure 19 The effect of volume of R-B solution on the sensitivity for determination of iron(III) using SIA system

4.5.1.3 Total aspirated volume of R-B solution

As excess of total reagent used that need to be studied to ensure complex formation completion. The aspirate volume of R-B can be affected to the reaction between iron(III) solution and betel nut extract to from iron(III)- betel nut complex. The effect of aspirated R-B volume on sensitivity was studied between 30 to 240 µL. The results are given in Table 33 and Figure 20. The sensitivity increased slowly from 30 to 120 µL of R-B solution. After that, sensitivity decreased and stabled up to 150 µL. A decreasing in sensitivity was observed using the aspiration volume more than 150 µL because de-mixing of reagent and iron was also occurred. Furthermore, the tailing peak of the reagent zone was observed. Hence, an aspirated volume at 120 µL of R-B was chosen as an optimum for experiments.

Table 33 The effect of aspirated volume of R-B solution on the absorbance of iron(III) complex

Volume of R-B (µL)	Absorbance		Net signal
	Blank	Iron(III) complex	
30	0.0206	0.0854	0.0657
	0.0202	0.0877	
	0.0208	0.0857	
$\bar{X}$ ±SD	0.0205±0.0003	0.0862±0.0012	
60	0.0226	0.0959	0.0730
	0.0221	0.0965	
	0.0239	0.0953	
$\bar{X}$ ±SD	0.0228±0.0009	0.0959±0.0006	
90	0.0243	0.1033	0.0790
	0.0248	0.1031	
	0.0252	0.1048	
$\bar{X}$ ±SD	0.0247±0.0005	0.1038±0.0009	
120	0.0291	0.1419	0.1127
	0.0303	0.1440	
	0.0304	0.1422	
$\bar{X}$ ±SD	0.0300±0.0007	0.1427±0.0011	
150	0.0485	0.1328	0.0888
	0.0471	0.1383	
	0.0474	0.1383	
$\bar{X}$ ±SD	0.0477±0.0007	0.1365±0.0032	
180	0.0509	0.1395	0.0891
	0.0512	0.1396	
	0.0505	0.1409	
$\bar{X}$ ±SD	0.0509±0.0004	0.1400±0.0008	
210	0.0541	0.1441	0.0897
	0.0546	0.1413	
	0.0544	0.1469	
$\bar{X}$ ±SD	0.0544±0.0003	0.1441±0.0028	
240	0.0670	0.1516	0.0843
	0.0667	0.1504	
	0.0656	0.1502	
$\bar{X}$ ±SD	0.0664±0.0008	0.1507±0.0007	

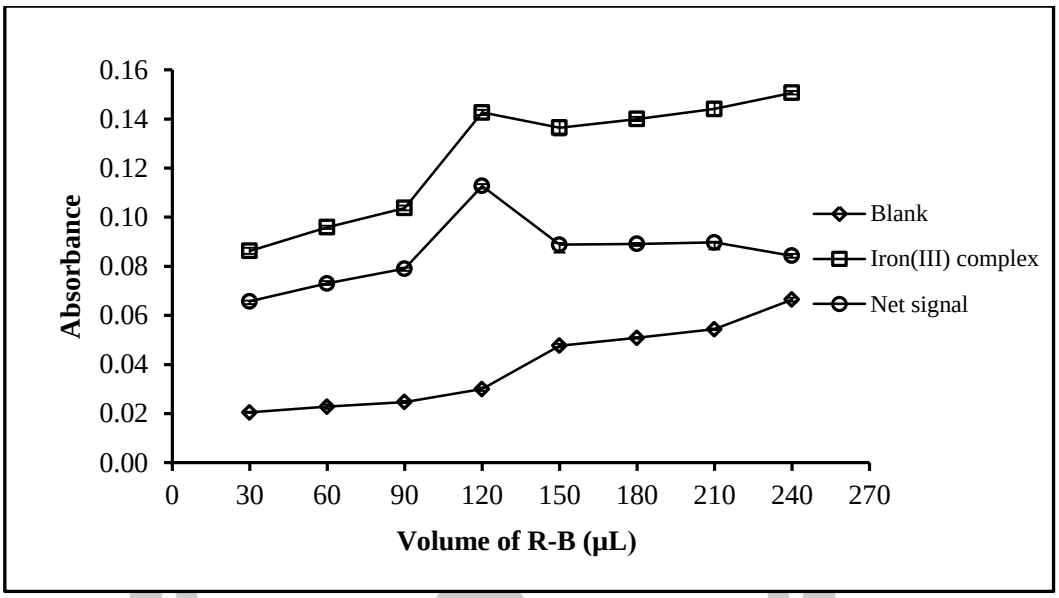


Figure 20 The effect of volume of R-B solution on the sensitivity for determination of iron(III) using SIA system

4.5.1.4 Total aspirated volume of iron(III) standard or sample solution

Employing the small sample volume can be affected the signal because low analyte concentration. However, using large of the sample volume might be caused incomplete reactions due to excessive analyte concentration. Therefore, the effect of total aspirated volume of solution standard or sample on the sensitivity of the system was studied in the ranging of 60 to 270 µL. The results are presented in Table 34 and Figure 21. It was observed that absorbance value increased with standard or sample volume increasing up to 180 µL and it remained almost constant. Therefore, 180 µL of standard or sample volume was chosen for this experiment because larger sample volumes needed to longer rinsing time, which results in reduced frequency of analysis and cannot increase the sensitivity.

Table 34 The effect of aspirated volume of standard or sample volume on the absorbance of iron(III) complex

Volume of standard or sample(mL)	Absorbance		Net signal
	Blank	Iron(III) complex	
60	0.0536	0.1119	0.0598
	0.0536	0.1159	
	0.0531	0.1119	
$\bar{X}$ ±SD	0.0534±0.0003	0.1132±0.002323	
90	0.0487	0.1279	0.0789
	0.0486	0.1277	
	0.0488	0.1272	
$\bar{X}$ ±SD	0.0487±0.0001	0.1276±0.0003	
120	0.0468	0.1423	0.0960
	0.0465	0.1426	
	0.0463	0.1426	
$\bar{X}$ ±SD	0.0465±0.0002	0.1425±0.0002	
150	0.0432	0.1463	0.1029
	0.0439	0.1466	
	0.0439	0.1468	
$\bar{X}$ ±SD	0.0436±0.0004	0.1466±0.0003	
180	0.0004	0.1511	0.1200
	0.0331	0.1536	
	0.0332	0.1547	
$\bar{X}$ ±SD	0.0331±0.00004	0.1532±0.0018	
210	0.0345	0.1466	0.1121
	0.0344	0.1465	
	0.0340	0.1462	
$\bar{X}$ ±SD	0.0343±0.0002	0.1464±0.0002	
240	0.0331	0.1454	0.1123
	0.0332	0.1460	
	0.0333	0.1452	
$\bar{X}$ ±SD	0.0332±0.00008	0.1455±0.0004	
270	0.0303	0.1429	0.1129
	0.0302	0.1431	
	0.0302	0.1434	
$\bar{X}$ ±SD	0.0302±0.00006	0.1431±0.0002	

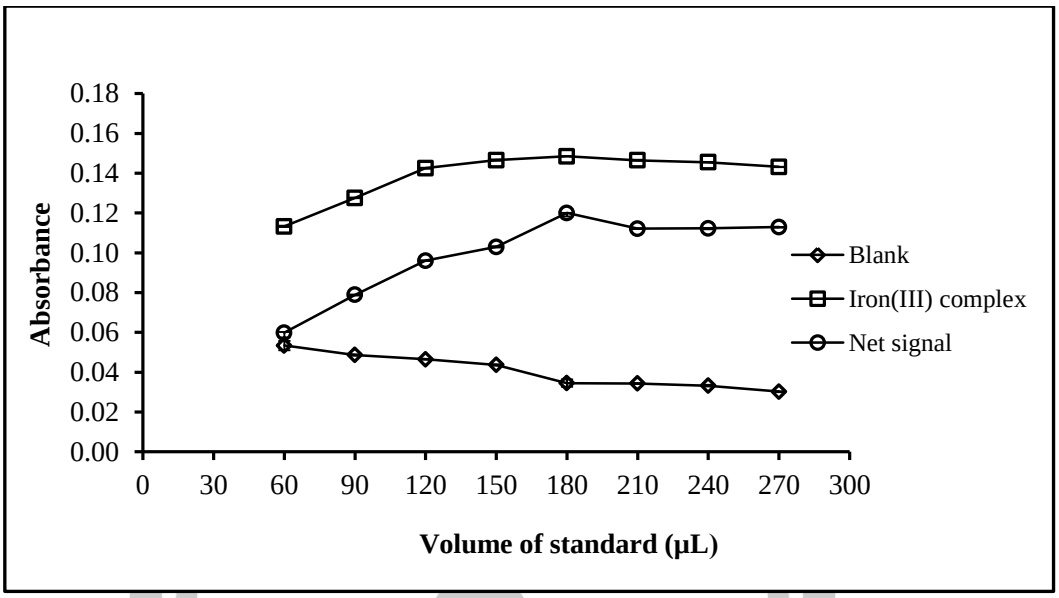


Figure 21 The effect of volume of standard on the sensitivity for determination of iron(III) using SIA system

4.5.1.5 Effect of reaction coil length

In flow-based system, the reaction coil length can be affected to the sensitivity and reaction time of analytical method. Employing short of the reaction coil, the reagent and analyte would react incompletely. But using long reaction coil, the reaction zone would be dispersed resulting to reduce sensitivity and it also more time consumption. Hence, the effects of reaction coil were studied different in length from 0-150 cm. As results shown in Table 35 and Figure 22**,** it was found that, the absorbance was increased as the reaction coil increasing up to 100 cm. Then, decreasing of sensitivity was observed when the length was over 100 cm due to the disseminate of the reaction zone in the reaction coil. Therefore, a reaction coil length of 100 cm was selected for the determination of iron(III) by the developed method.

Table 35 The effect of reaction coil length of standard or sample volume on the absorbance of iron(III) complex

Reaction coil length (cm)	Absorbance		Net signal
	Blank	Iron(III) complex	
0	0.0199	0.1448	0.1239
	0.0198	0.1439	
	0.0195	0.1421	
$\bar{X}$ ±SD	0.0197±0.0002	0.1436±0.0014	
50	0.0217	0.1510	0.1289
	0.0215	0.1507	
	0.0218	0.1500	
$\bar{X}$ ±SD	0.0217±0.0002	0.1506±0.0005	
100	0.0250	0.1639	0.1400
	0.0243	0.1652	
	0.0244	0.1646	
$\bar{X}$ ±SD	0.0246±0.0004	0.1646±0.0066	
150	0.0276	0.1460	0.1186
	0.0278	0.1464	
	0.0280	0.1467	
$\bar{X}$ ±SD	0.0278±0.0002	0.1464±0.0004	

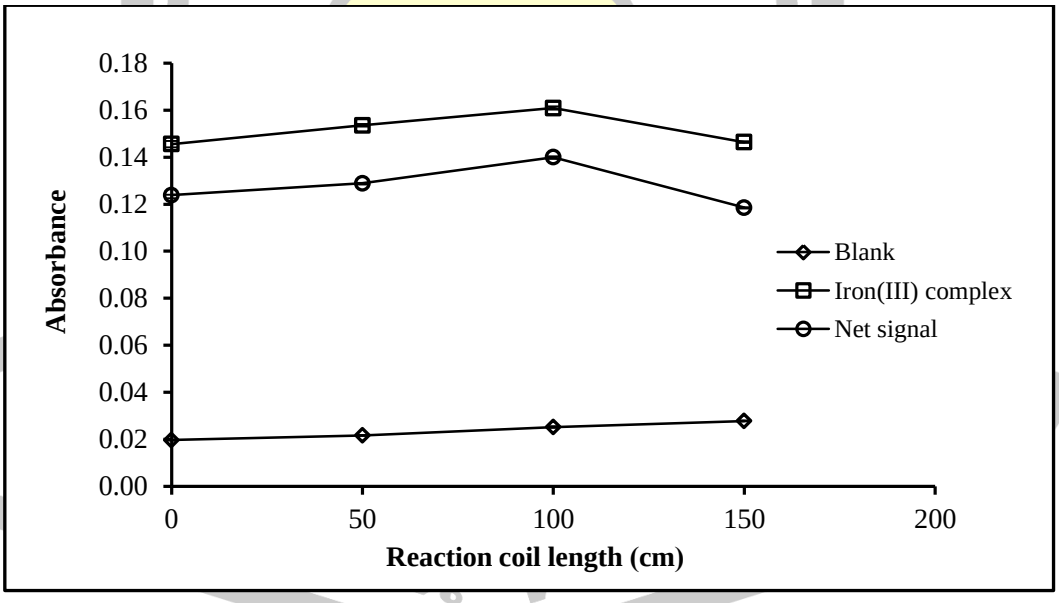


Figure 22 The effect of reaction coil length on the sensitivity for determination of iron(III) using SIA system

4.5.1.6 Effect of dispensing flow rate

The effect of dispensing flow rate to deriver of the reaction product to the detector had significantly effected on the absorption signal of iron(III)- betel nut complex. The dispensing flow rate was investigated in the range 30-110 µL s^{-1}. Utilizing high flow rate leads to shorter time for either sample passing through the flow through-cell, a low signal was obtained. While, a lower flow rate used, the residence time for either sample is long and dispersion in large which can be reduced the sensitivity and sample throughput. The result is illustrated in Table 36 and Figure 23, flow rates over 50 µL s^{-1} cause's sensitivity to decrease. Then, the signal was leveled off because the product was produced in short response time at high flow rates. Utilizing dispensing flow rates lower than 50 µL s^{-1} resulted in dispersion of mixing zone and causes the signal to decrease. Hence, a dispensing flow rate as 50 µL s^{-1} was selected. The sample throughput was 40 h^{-1} using dispensing flow rate at 50 µL s^{-1}.

Table 36 The effect of dispensing flow rate of standard or sample volume on the absorbance of iron(III) complex

Flow rate (µL s^{-1})	Absorbance		Net signal
	Blank	Iron(III) complex	
30	0.0245	0.1302	0.1056
	0.0247	0.1302	
	0.0249	0.1307	
$\bar{X}$ ±SD	0.0247±0.0002	0.1304±0.0003	
50	0.0170	0.1339	0.1164
	0.0170	0.1331	
	0.0170	0.1332	
$\bar{X}$ ±SD	0.0170±0.00002	0.1334±0.0005	
70	0.0174	0.1216	0.1039
	0.0173	0.1215	
	0.0179	0.1213	
$\bar{X}$ ±SD	0.0175±0.0003	0.1215±0.0001	
90	0.0273	0.1307	0.1032
	0.0273	0.1309	
	0.0274	0.1302	
$\bar{X}$ ±SD	0.0273±0.00005	0.1306±0.0004	
110	0.035	0.136	0.1010
	0.034	0.136	
	0.035	0.135	
$\bar{X}$ ±SD	0.0346±0.0005	0.1356±0.0005	

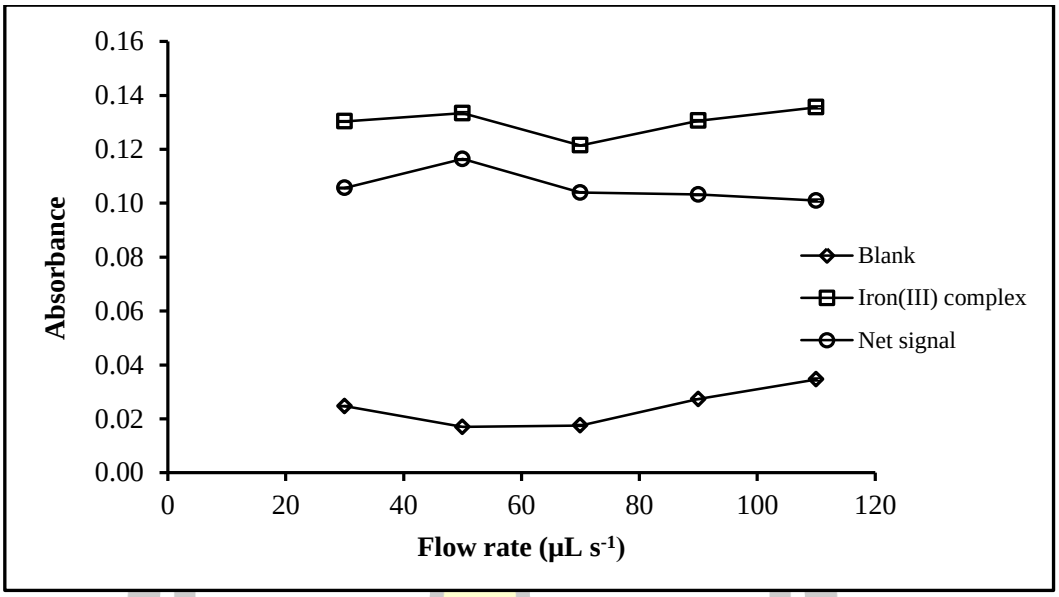


Figure 23 The effect of flow rate on the sensitivity for determination of iron(III) using SIA system.

4.5.1.7 The different source of betel nut material

The effect different sources of betel nut from local fresh market in Roi-Et province, local fresh market in Maha Sarakham province and local fresh market in Bangkok province, Thailand was investigated. As the results was shown in Table 37 and Figure 24. It was found that, betel nut purchased from different sources was not significantly given different of absorbance. Therefore, different source reagents can be used together in the experiments. However, to avoid the variation of absorption signal, the calibration was studied in everyday.

Table 37 The effect of on different source the absorbance of iron(III) complex

Difference source	Absorbance		Net signal
	Blank	Iron(III) complex	
1	0.0389	0.1511	0.1131
	0.0382	0.1517	
	0.0381	0.1515	
$\bar{X}$ ±SD	0.0384±0.0004	0.1515±0.0003	
2	0.0690	0.1865	0.1178
	0.0687	0.1866	
	0.0683	0.1865	
$\bar{X}$ ±SD	0.0687±0.0003	0.1865±0.00007	
3	0.0332	0.1446	0.1112
	0.0334	0.1447	
	0.0337	0.1448	
$\bar{X}$ ±SD	0.0335±0.0003	0.1447±0.0001	
4	0.0631	0.1801	0.1173
	0.0631	0.1807	
	0.0630	0.1802	
$\bar{X}$ ±SD	0.0631±0.00005	0.1804±0.0003	
5	0.0497	0.1611	0.1118
	0.0496	0.1618	
	0.0492	0.1610	
$\bar{X}$ ±SD	0.0495±0.0003	0.1613±0.0005	
6	0.0423	0.1596	0.117589
	0.0422	0.1599	
	0.0420	0.1597	
$\bar{X}$ ±SD	0.0422±0.0002	0.1597±0.0002	
7	0.0368	0.1479	0.1107
	0.0370	0.1473	
	0.0369	0.1474	
$\bar{X}$ ±SD	0.0369±0.0001	0.1475±0.0003	
8	0.0385	0.1491	0.1109
	0.0390	0.1497	
	0.0384	0.1497	
$\bar{X}$ ±SD	0.0386±0.00003	0.1495±0.0004	

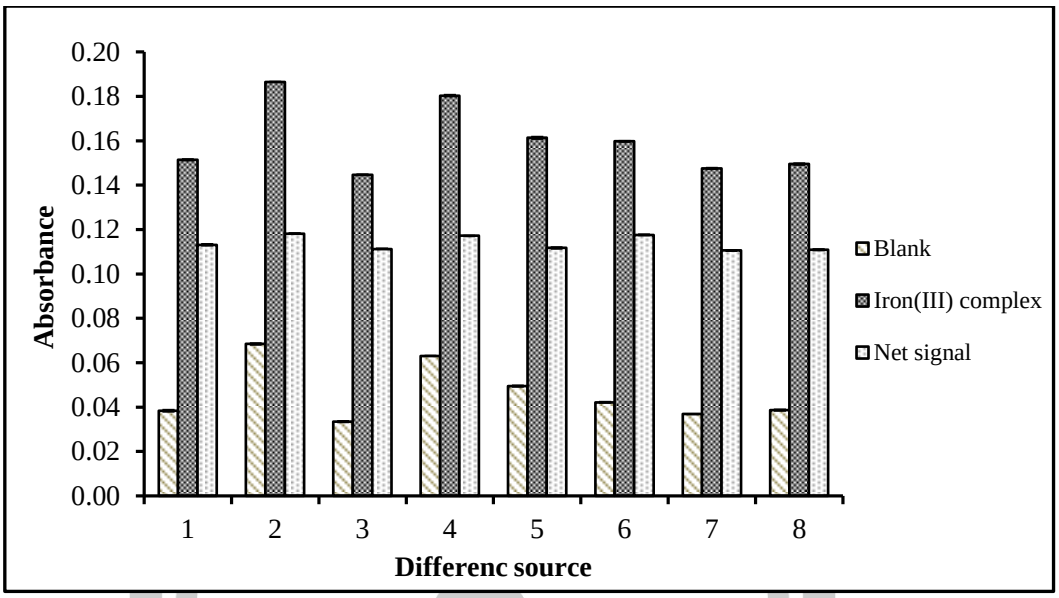


Figure 24 The effect of different source of plant materials (1)-(4) local fresh market in Roi-Et province; (5) local fresh market in Mahasarakham province; (6)-(7) local fresh market in Bangkok province and (8) Planting at home

4.5.1.8 The stability of the betel nut extracts solution

In general, plant extract fast decompose by expose to air, so the stability of betel nut extract was studied. The solution of the betel nut (0.5 g in 100 mL) was prepared and was kept in polyethylene bottle at room temperature for 30 min-72 hours**.** The stability of natural reagent extracted solution was examined by mixing with iron(III) solution in acetate buffer pH 5.5. Absorbance value was analyzed 565 nm in every hour. It was found that the absorbance signal of the complex was not significant different after stored for 48 hour as shown in Table 38 and Figure 25. Therefore, betel nut extracted solution is very stable and the reagent is more suitable for SIA experiments. However, the extract solution should be freshly prepared.

Table 38 The effect of stability of the betel nut extracts solution on the absorbance of iron(III) complex

Time of stability (hours)	Absorbance		Net signal
	Blank	Iron(III) complex	
0.5	0.0376	0.1404	0.1030
	0.0371	0.1404	
	0.0375	0.1403	
$\bar{X}$ ±SD	0.0374±0.0003	0.1404±0.00005	
1	0.0377	0.1403	0.1031
	0.0376	0.1408	
	0.0371	0.1406	
$\bar{X}$ ±SD	0.0375±0.0003	0.1406±0.0003	
2	0.0377	0.1419	0.1038
	0.0379	0.1413	
	0.0376	0.1412	
$\bar{X}$ ±SD	0.0377±0.0002	0.1415±0.0004	
4	0.0379	0.1406	0.1030
	0.0373	0.1407	
	0.0375	0.1406	
$\bar{X}$ ±SD	0.0376±0.0003	0.1406±0.0004	
6	0.0499	0.1538	0.1039
	0.0495	0.1534	
	0.0496	0.1537	
$\bar{X}$ ±SD	0.0497±0.0002	0.1536±0.0002	
9	0.0511	0.1547	0.1036
	0.0513	0.1550	
	0.0511	0.1548	
$\bar{X}$ ±SD	0.0512±0.0001	0.1548±0.0001	
12	0.0502	0.1561	0.1054
	0.0507	0.1561	
	0.0560	0.1560	
$\bar{X}$ ±SD	0.0506±0.0004	0.1561±0.00005	
24	0.0625	0.1690	0.1046
	0.0652	0.1673	
	0.0639	0.1693	
$\bar{X}$ ±SD	0.0639±0.0013	0.1685±0.0011	
48	0.0698	0.1714	0.1048
	0.0635	0.1736	
	0.0689	0.1714	
$\bar{X}$ ±SD	0.0674±0.0034	0.1722±0.0013	
72	0.0794	0.1708	0.0924
	0.0784	0.1704	
	0.0769	0.1706	
$\bar{X}$ ±SD	0.0783±0.0012	0.1706±0.0002	

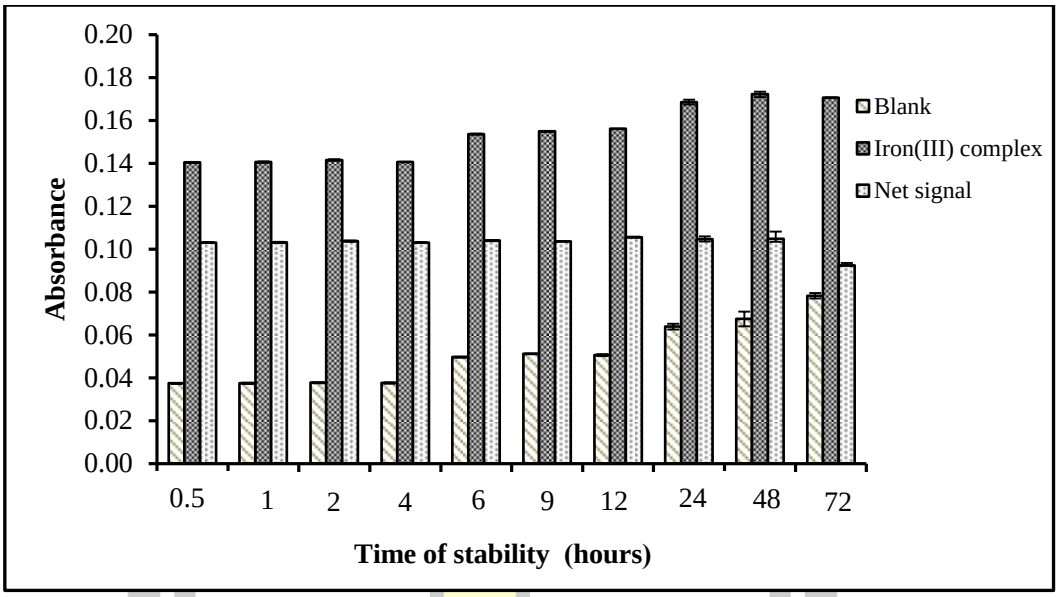


Figure 25 The effect of stability of natural reagent extracts solution

4.5.2 The optimum conditions for determination of ascorbic acid

An indirect proposed method SIA for ascorbic acid determination is based on the redox-reaction to reduce iron(III) to iron(II) by ascorbic acid as it is described in equation 10 (Elmagirbi et al. 2012)

$$2Fe^{3+} + C_6H_8O_6 \longrightarrow 2Fe^{2+} + 2H^{+} + C_6H_6O_6 \quad \ldots\ldots\ldots\ldots 10$$

After, reducing iron(III) to iron(II) by ascorbic acid, the absorbance at 565 nm was decreased, due to the concentration of with iron(III) was decreased as the absorption spectrum shown in Figure 26.

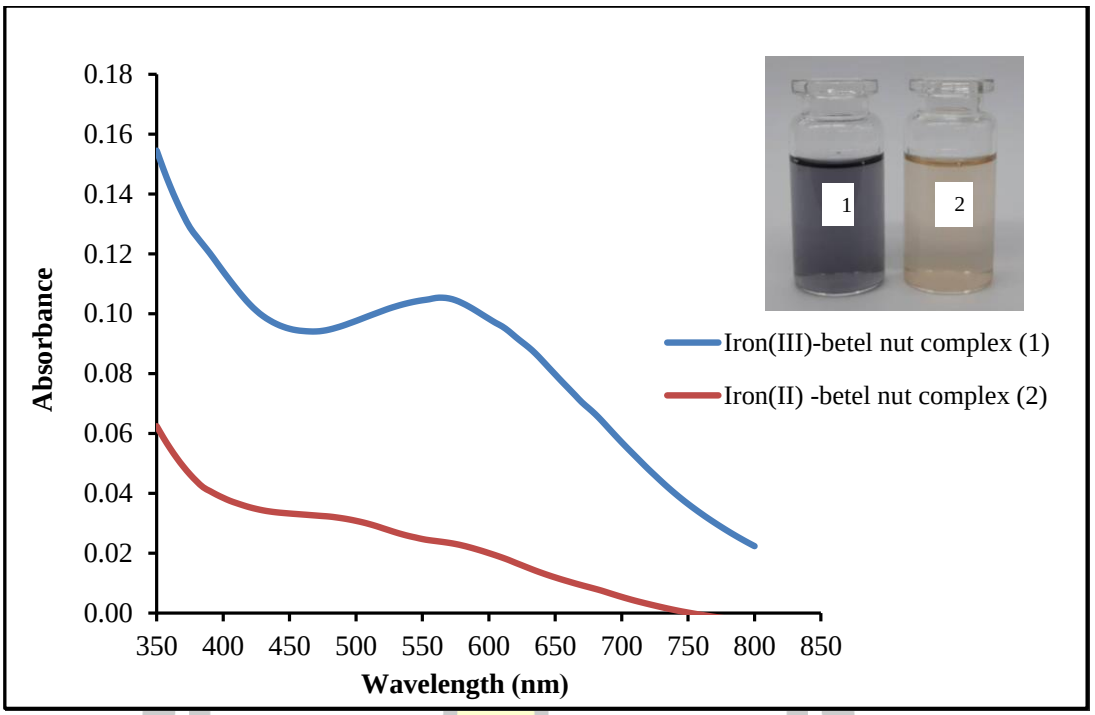


Figure 26 Absorption spectra of indirect for determination of ascorbic acid, Conditions; 10 mg L^{-1} of iron(III), 20 mg L^{-1} of ascorbic acid and 5 mL of 0.7 M acetate buffer

4.5.3 Optimization of SIA system for determination of ascorbic acid

Physical and chemical variables were optimized for the proposed SI method. This study was conducted by changing each variable while keeping others stable. Various parameters such as concentration and aspiration volume of iron(III), volume of standard ascorbic acid/sample, volume of R-B solution, mixing coil length and flow rate were investigated for the determination of ascorbic acid.

4.5.3.1 Effect of aspiration sequence profile

Effect of aspirated sequential profiles was investigated using a total volume of 300 µL. Different segment profiles were demonstrated. The optimum sequence order was shown in Table 39.

Table 39 The different sequence profiles for determination of ascorbic acid

Sequence No.	Sequence order aspiration	Volume (µL)
1	R-B/Iron(III)/SD/R-B/SD/Iron(III)/SD/ R-B/SD/Iron(III)/R-B	20/40/25/20/25/40/25/20/25/40/20
2	R-B/SD/Iron(III)/R-B/SD/Iron(III)/SD/ R-B/SD/Iron(III)/R-B	20/25/40/20/25/40/25/20/25/40/20
3	R-B/SD/Iron(III)/SD/R-B SD/Iron(III)/SD/R-B/SD/Iron(III)/ SD/R-B	20/10/40/20/20/20/40/20/20/20/40/10/20

Table 40 The effect of different sequence on the absorbance of ascorbic acid

Sequence No.	Absorbance		Net signal
	Blank	Iron(II) complex	
1	0.0880	0.0708	0.0172
	0.0860	0.0704	
	0.0891	0.0703	
$\bar{X}$ ±SD	0.0877±0.0016	0.0705±0.0003	
2	0.0733	0.0624	0.0104
	0.0738	0.0655	
	0.0717	0.0635	
$\bar{X}$ ±SD	0.0742±0.0028	0.0638±0.0016	
3	0.0523	0.0427	0.0097
	0.0532	0.0441	
	0.0512	0.0409	
$\bar{X}$ ±SD	0.0522±0.0010	0.0.426±0.0016	

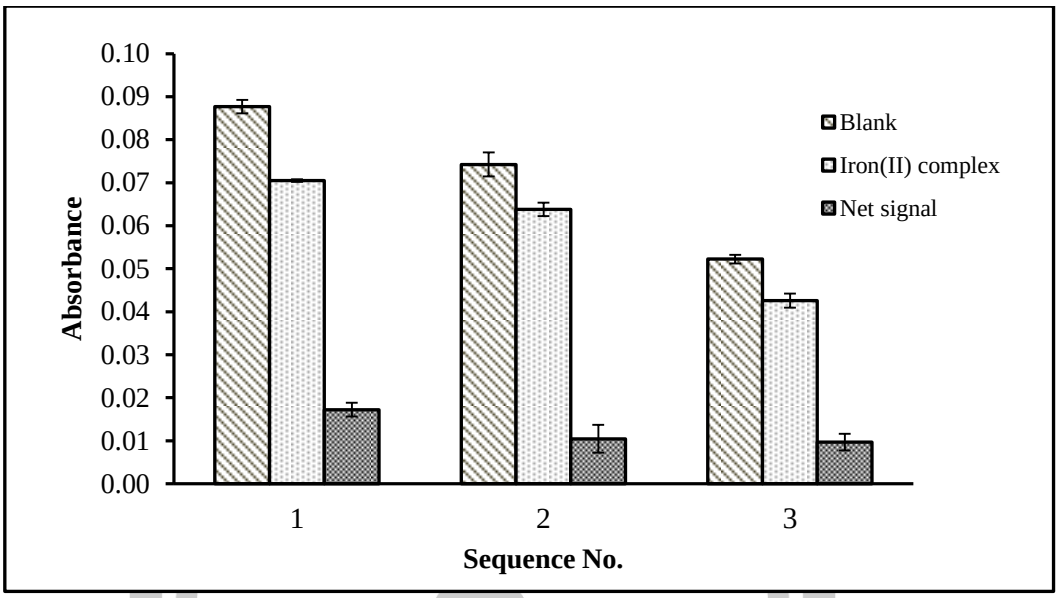


Figure 27 Investigation of sequence profiles for SIA system

The results found that the aspirated segment No.1 was provided the highest absorbance as shown in Table 40 and Figure 27. Therefore, the suitable aspiration sequence for the proposed method was R-B/Iron(III)/SD/R-B/SD/Iron(III)/SD/R-B/SD/Iron(III)/R-B. So, segment 1 was utilized in further experiments**.**

4.5.3.2 Effect of iron(III) concentration

In order to examine the influence of iron(III) concentration solution, a difference iron(III) concentration from 10 to 50 mg L^{-1} were tested on the determination of 50 mg L^{-1} ascorbic acid. The absorbance was decreased with increasing the concentration of iron(III) solution up to 40 mg L^{-1}. There was no changed in adsorption when the amount of iron(III) increased to 50 mg L^{-1} as shown in Table 41 and Figure 28. This indicates that the reaction was entered an equilibrium state. And iron(III) was completely reduced to iron(II). So, 40 mg L^{-1} of iron(III) was selected as the optimal values.

Table 41 The effect of iron(III) concentration on the absorbance of ascorbic acid

Concentration of iron(III)	Absorbance		Net signal
	Blank	Iron(II) complex	
10	0.0990	0.0899	0.0112
	0.0986	0.0860	
	0.0999	0.0880	
$\bar{X}$ ±SD	0.0992±0.0007	0.0879±0.0019	
15	0.1121	0.0903	0.0196
	0.1122	0.0925	
	0.1124	0.0950	
$\bar{X}$ ±SD	0.1122±0.00019	0.0926±0.0024	
20	0.1351	0.1104	0.0213
	0.1358	0.1108	
	0.1351	0.1209	
$\bar{X}$ ±SD	0.1353±0.0004	0.1140±0.0060	
30	0.1604	0.1273	0.0324
	0.1597	0.1291	
	0.1604	0.1 269	
$\bar{X}$ ±SD	0.1602±0.0004	0.1278±0.0060	
35	0.1920	0.1588	0.0373
	0.1921	0.1501	
	0.1929	0.1562	
$\bar{X}$ ±SD	0.1923±0.0005	0.1550±0.0045	
40	0.2229	0.1764	0.0438
	0.2226	0.1796	
	0.2151	0.1732	
$\bar{X}$ ±SD	0.2202±0.0044	0.1764±0.0032	
50	0.2384	0.1960	0.0431
	0.2388	0.1945	
	0.2388	0.1962	
$\bar{X}$ ±SD	0.2387±0.00026	0.1956±0.00091	

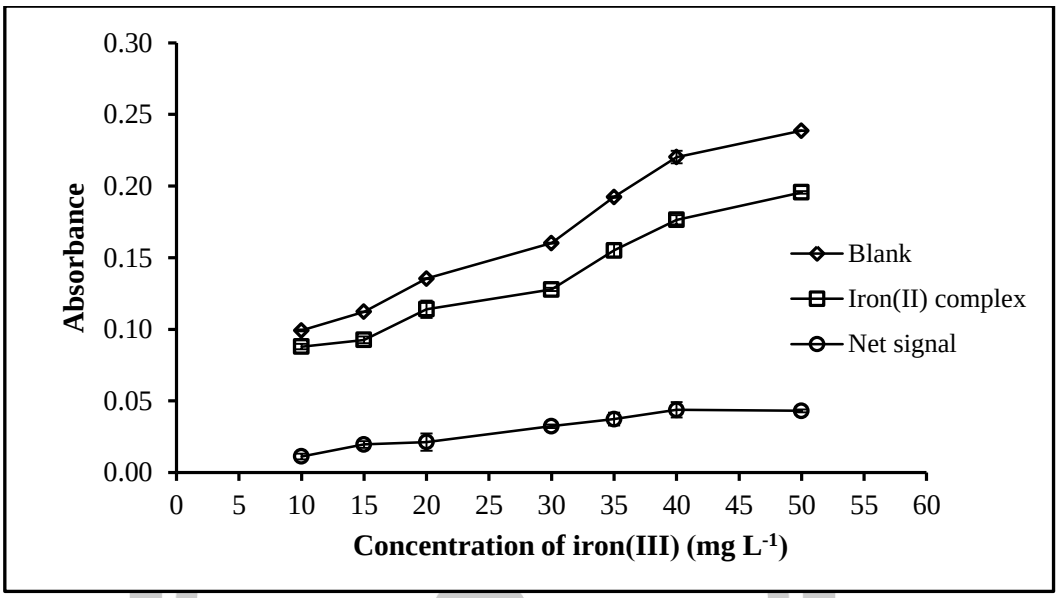


Figure 28 Effect of concentration of iron(III) on the determination of ascorbic acid

4.5.3.3 Aspirate volume of iron(III) solution/ R-B solution/ ascorbic acid standard or sample solution

The aim of optimizing is to minimize the volume of iron(III)/R-B reagent/ ascorbic acid standard or sample that provided sensitivity and high reproducibility.

The higher aspiration volume of the solution was lead to longer mixing time. A suitable volume of solution can be affected to the mixing and forming complex, resulting in higher absorbance. The aspirated volume of iron(III) was studied from 60-180 µL, the standard ascorbic acid or sample volume was investigated from 60-120 µL and the R-B reagent volume was tested from 60-120 µL. The result presented in Table 42, 43 and 44, Figure 29, 30 and 31, respectively. Therefore, the injected sample/standard volume was used at 100 µL. While, optimal reagent volume was 80 µL and iron(III) volume was 120 µL.

Table 42 The effect of iron(III) solution on the absorbance of ascorbic acid

Volume of iron(III) solution (µL)	Absorbance		Net signal
	Blank	Iron(II) complex	
60	0.0491	0.0355	0.0155
	0.0495	0.0332	
	0.0491	0.0325	
$\bar{X}$ ±SD	0.0492±0.0002	0.0337±0.0016	
120	0.0742	0.0402	0.0330
	0.0744	0.0406	
	0.0741	0.0428	
$\bar{X}$ ±SD	0.0742±0.0002	0.0412±0.0014	
180	0.0788	0.0475	0.0323
	0.0784	0.0451	
	0.0783	0.0461	
$\bar{X}$ ±SD	0.0785±0.0003	0.0462±0.0012	

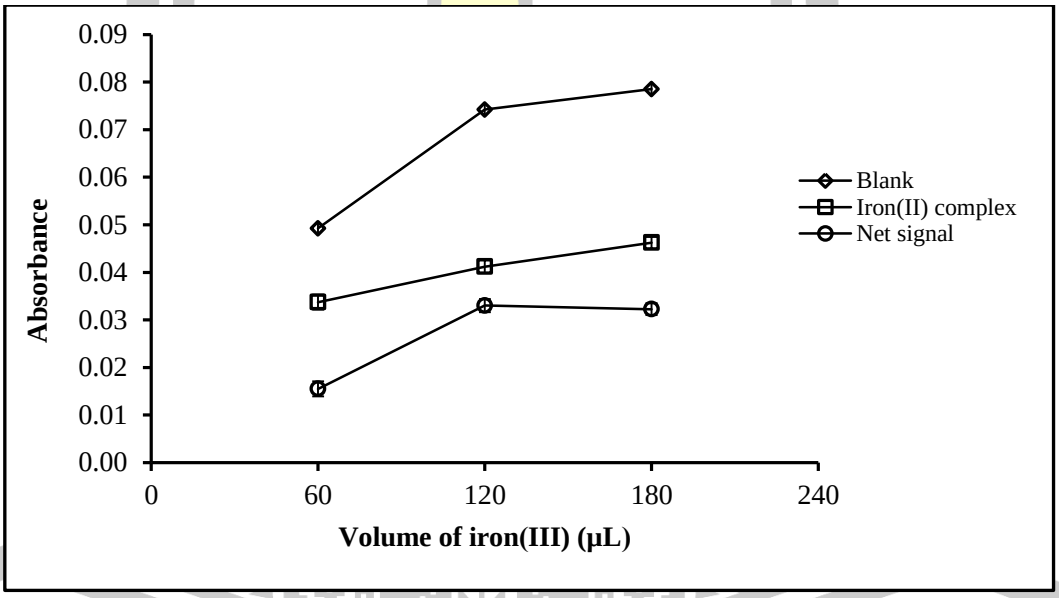


Figure 29 Effect of the aspiration volume of iron(III) solution on the determination of ascorbic acid

Table 43 The effect of R-B solution on the absorbance of ascorbic acid

Volume of R-B solution (µL)	Absorbance		Net signal
	Blank	Iron(II) complex	
60	0.0551	0.0416	0.0112
	0.0555	0.0460	
	0.0544	0.0438	
$\bar{X}$ ±SD	0.0550±0.0006	0.0438±0.0022	
80	0.0626	0.0309	0.0311
	0.0627	0.0315	
	0.0629	0.0325	
$\bar{X}$ ±SD	0.0627±0.0001	0.0316±0.0008	
100	0.0689	0.0491	0.0179
	0.0687	0.0509	
	0.0676	0.0515	
$\bar{X}$ ±SD	0.0684±0.0007	0.0505±0.0012	
120	0.0763	0.0610	0.0157
	0.0763	0.0597	
	0.0761	0.0610	
$\bar{X}$ ±SD	0.0762±0.0001	0.0605±0.0007	

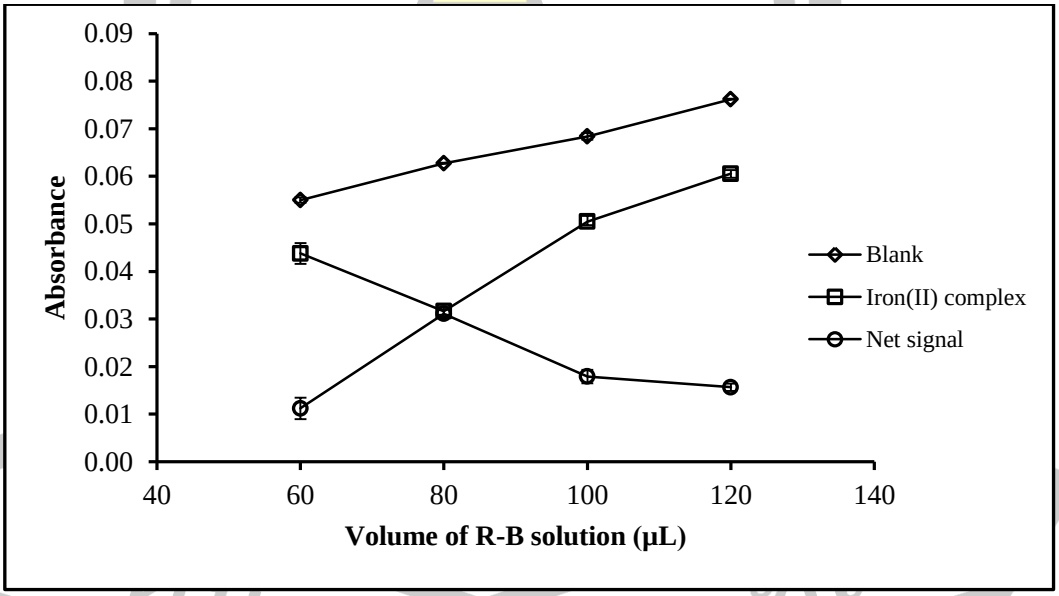


Figure 30 Effect of the aspiration volume of R-B solution on the determination of ascorbic acid

Table 44 The effect of standard solution on the absorbance of ascorbic acid

Volume Standard or sample solution (µL)	Absorbance		Net signal
	Blank	Iron(II) complex	
60	0.0577	0.0479	0.0102
	0.0578	0.0494	
	0.0578	0.0455	
$\bar{X}$ ±SD	0.0578±0.00004	0.0476±0.0019	
80	0.0613	0.0379	0.0258
	0.0618	0.0325	
	0.0603	0.0356	
$\bar{X}$ ±SD	0.0612±0.0008	0.0353±0.0027	
100	0.0731	0.0446	0.0313
	0.0736	0.0405	
	0.0738	0.0414	
$\bar{X}$ ±SD	0.0735±0.00003	0.0422±0.0021	
120	0.0787	0.0503	0.0279
	0.0792	0.0491	
	0.0790	0.0538	
$\bar{X}$ ±SD	0.0790±0.0002	0.0511±0.0024	

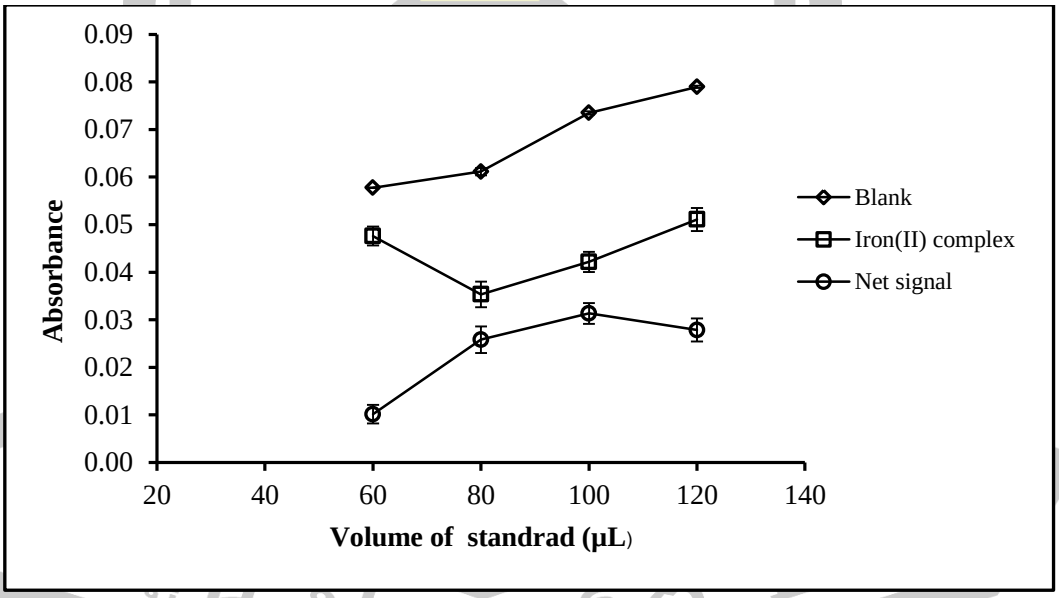


Figure 31 Effect of the aspiration volume of standard solution on the determination of ascorbic acid

4.5.3.4 Effect of reaction coil length

The effect of the reaction coils length was examined in the ranging of 0-150 cm. The results indicated that the net signal was increased with increasing mixing coil length up to 100 cm. By increasing the reaction coil length above 100 cm absorbance was decreased. Hence, 100 cm was chosen as the optimal reaction coil length as shown in Table 45 and Figure 32.

Table 45 The effect of coil length on the absorbance of ascorbic acid

Reaction coil length (cm)	Absorbance		Net signal
	Blank	Iron(II) complex	
0	0.0580	0.0537	0.0051
	0.0588	0.0544	
	0.0573	0.0507	
$\bar{X}$ ±SD	0.0580±0.0008	0.0529±0.0019	
50	0.0628	0.0500	0.0146
	0.0602	0.0461	
	0.0616	0.0448	
$\bar{X}$ ±SD	0.0615±0.0013	0.0469±0.0027	
100	0.0729	0.0355	0.0395
	0.0716	0.0301	
	0.0719	0.0323	
$\bar{X}$ ±SD	0.0721±0.0007	0.0326±0.0030	
150	0.0731	0.0401	0.0302
	0.0735	0.0446	
	0.0744	0.0458	
$\bar{X}$ ±SD	0.0737±0.0007	0.0435±0.0030	

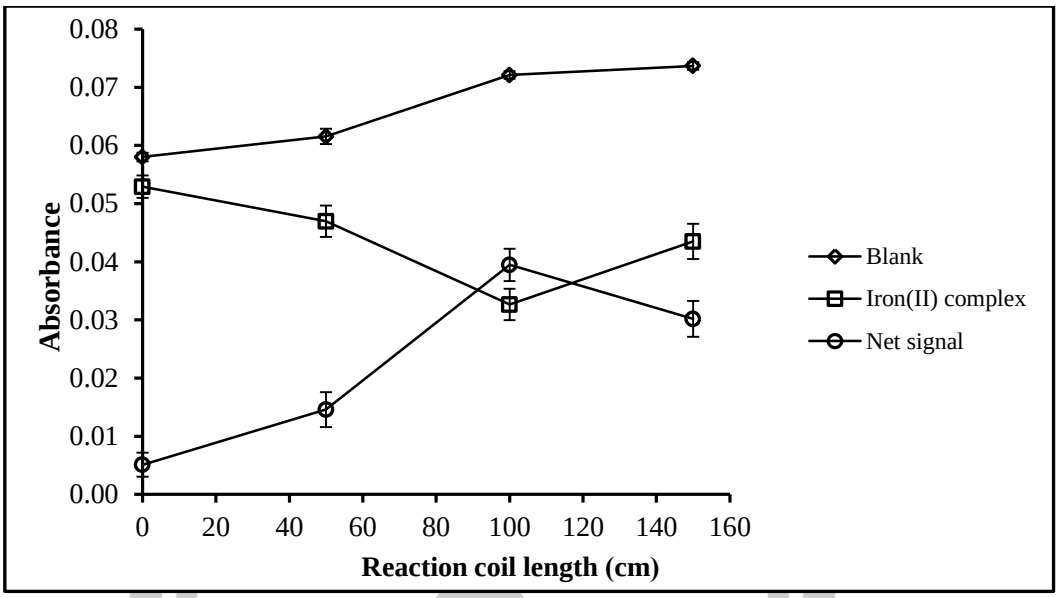


Figure 32 Effect of coil length on the determination of ascorbic acid

4.5.3.5 Effect of dispensing flow rate

The flow rate is very importance parameter that to be optimized because it influences to the product complex. The flow rate was investigated in the range from 30-110 µL s^{-1}. The optimum value chosen was 70 µL s^{-1} as shown in Table 46 and Figure 33. Employing higher flow rates, the analytical response was no significant a broad peak with long tail was obtained during slower flow rate.

Table 46 The effect of flow rate on the absorbance of ascorbic acid

Flow rate (µL s^{-1})	Absorbance		Net signal
	Blank	Iron(II) complex	
30	0.0608	0.0460	
	0.0624	0.0453	0.0156
	0.0617	0.0468	
$\bar{X}$ ±SD	0.0534±0.0081	0.0461±0.0008	
50	0.0701	0.0449	0.0274
	0.0717	0.0420	
	0.0714	0.0442	
$\bar{X}$ ±SD	0.0711±0.0009	0.0437±0.0015	
70	0.0753	0.0381	0.0361
	0.0754	0.0400	
	0.0750	0.0393	
$\bar{X}$ ±SD	0.0752±0.0002	0.0391±0.0010	
90	0.0695	0.0477	0.0212
	0.0690	0.0490	
	0.0697	0.0480	
$\bar{X}$ ±SD	0.0694±0.0003	0.0482±0.0007	
110	0.0674	0.0500	0.0162
	0.0674	0.0524	
	0.0678	0.0515	
$\bar{X}$ ±SD	0.0676±0.0002	0.0513±0.0012	

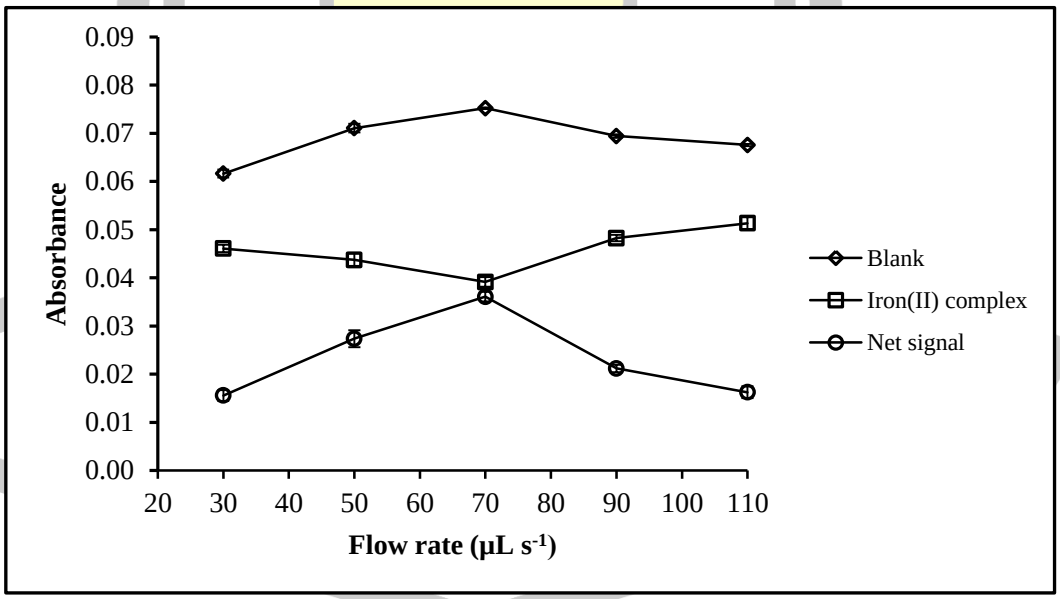


Figure 33 Effect of sample flow rate on the reaction product of ascorbic acid

4.6 Analytical figures of merit

4.6.1 Determination of iron(III)

The validation of the proposed method including the linearity range, LOD, LOQ, precision and accuracy were investigated. Under the optimum conditions as presented in Table 47**,** the linearity range for the calibration graph of iron(III) was achieved in the range 0.2-10 mg L^{-1}. The linear regression equation was y=0.0141x-0.0023 (r^2 =0.9995), where y is the absorbance of iron(III)- betel nut complex and x is the concentration of iron in mg L^{-1}. Figure 34 showed the SI signal profiles and calibration graph for the determination of iron (III) by developed method. LOD (3σ/s) and LOQ (10σ/s) (where σ is the standard deviation of reagent blank (n=10) and s is the slope of calibration curve) were 0.06 and 0.20 mg L^{-1}, respectively. The precision of the proposed method in terms of repeatability and reproducibility was measured using 10 replicates of four concentration of iron(III) solution at 0.3 ,3, 5 and 7 mg L^{-1}. It was found that, the %RSD of four studied concentration levels for repeatability were 3.22, 3.37, 2.26 and 0.70 (*n=10*), respectively and the reproducibility were 4.84, 4.93, 4.88 and 3.78 respectively (*n = 10*, 6 days) as summarized in the Table 48 of analytical performance for determination of iron(III).

Table 47 Optimum condition of the proposed method for iron(III) determination

Parameter	**Studied range**	**Optimum value**
Maximum absorption wavelength (nm)	350-800	565
pH	4.5-10	5.5
Concentration of pH(M)	0.1-1	0.7
Weight of reagent (g)	0.1-5	0.5
Extraction time(min)	10–60	20
Type of solvent	–	Hot water
Aspiration sequence		
sample(s), natural reagent with buffer solution (R-B)	segment order 1-5	segment order 4
Aspiration volume of solution (µL)		
Sample and/or standard iron(III)	60-270	180
Reagent solution (R-B)	30-240	120
Natural reagent of volume in 25 mL (mL)	0.5-20	7
Reaction coil length (cm)	0-150	100
Flow rate (µL s^{-1})	30-110	50

Table 48 Analytical performance for determination of iron(III) by developed method

Linear range (mg L^{-1})	0.2-10
Linear equation	y=0.0141x-0.0023
R^2	0.9995
Limit of detection (mg L^{-1})	0.06
Limit of quantification (mg L^{-1})	0.2
Sample rate (h^{-1})	40

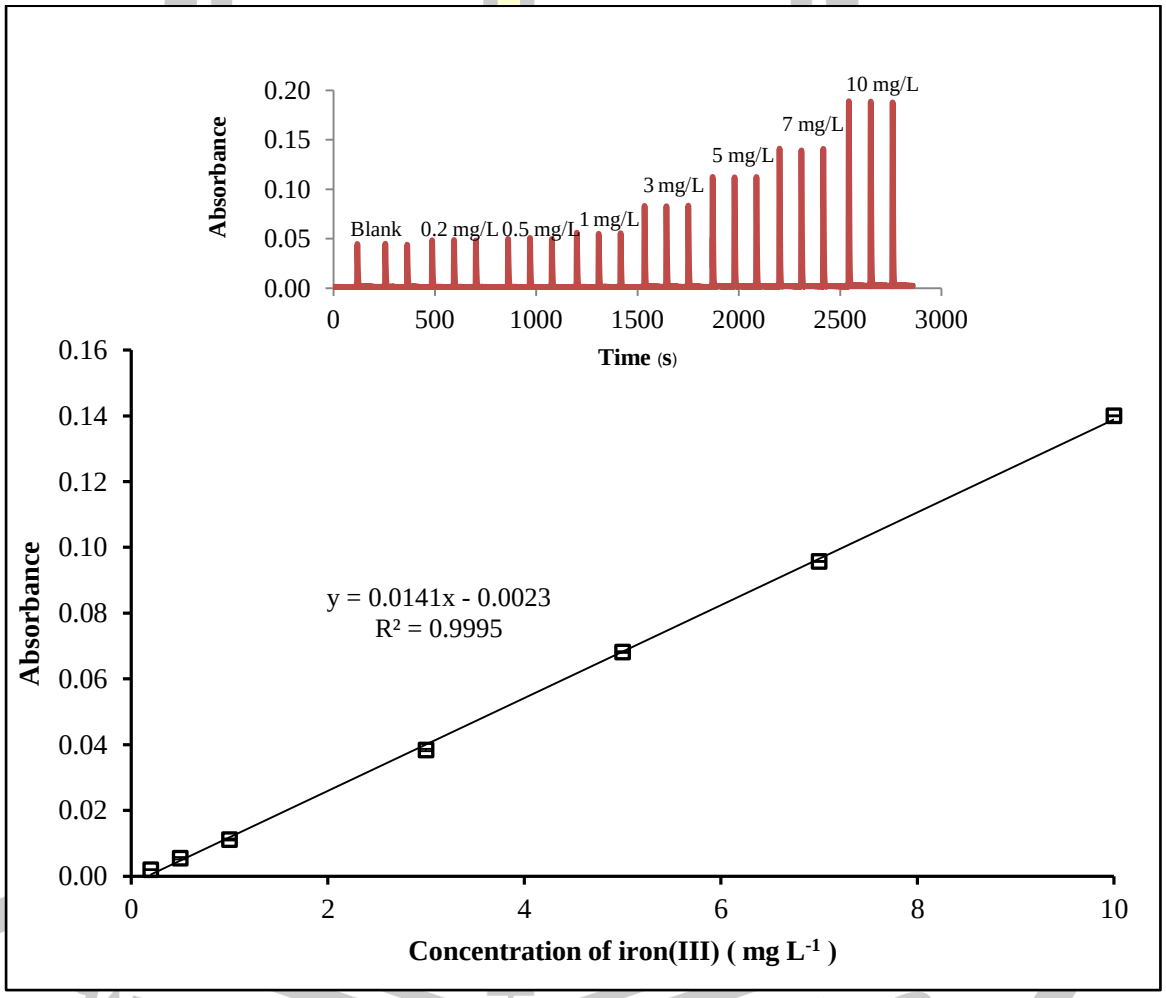


Figure 34 Calibration curve of iron(III) at 565 nm using the proposed method and SI gram of the iron(III) standard at concentration of iron(III)

4.6.2 Determination of ascorbic acid

The validation of the proposed method including the linearity range, LOD, LOQ, precision and accuracy were investigated. Under the optimum conditions as presented in Table 49**,** the linearity range for the calibration graph of ascorbic acid was achieved in the range 4-50 mg L^{-1} with a correlation coefficient of 0.9981. Calibration graph obeyed the equation: y=0.0031x+0.0039**,** where y is the absorbance and x is the concentration of ascorbic acid in mg L^{-1}. Figure 35 showed the SI signal profiles and calibration graph for the determination of ascorbic acid by developed method**.** LOD (3σ/*s*) and LOQ (10σ/*s*) (where σ is the standard deviation of reagent blank (*n*=10) and *s* is the slope of calibration curve) were 1.20 and 4.00 mg L^{-1}, respectively. The precision of the proposed method in terms of repeatability and reproducibility was measured using 10 replicates of two concentration of ascorbic acid solution at 10 and 20 mg L^{-1}. It was found that, the %RSD of two studied concentration levels for repeatability were 3.44 and 0.52 (*n=10*), respectively and the reproducibility were 4.72 and 1.39 respectively (*n = 10*, 5 days) as summarized in the Table 50 of analytical performance for determination of ascorbic acid.

Table 49 Optimization of manifold parameters and experiment condition of the proposed method for determination of ascorbic acid

Parameter	**Studied range**	**optimum value**
Aspiration segment order	segment order 1-3	segment 1
Aspiration volume of solution (µL)		
Sample and/or standard ascorbic acid	60-120	100
R-B solution	60-120	80
Iron(III) solution	60-180	120
Concentration of iron(III) (mg L^{-1})	10– 50	40
Reaction coil length (cm)	0-150	100
Flow rate (µL s^{-1})	30-110	70

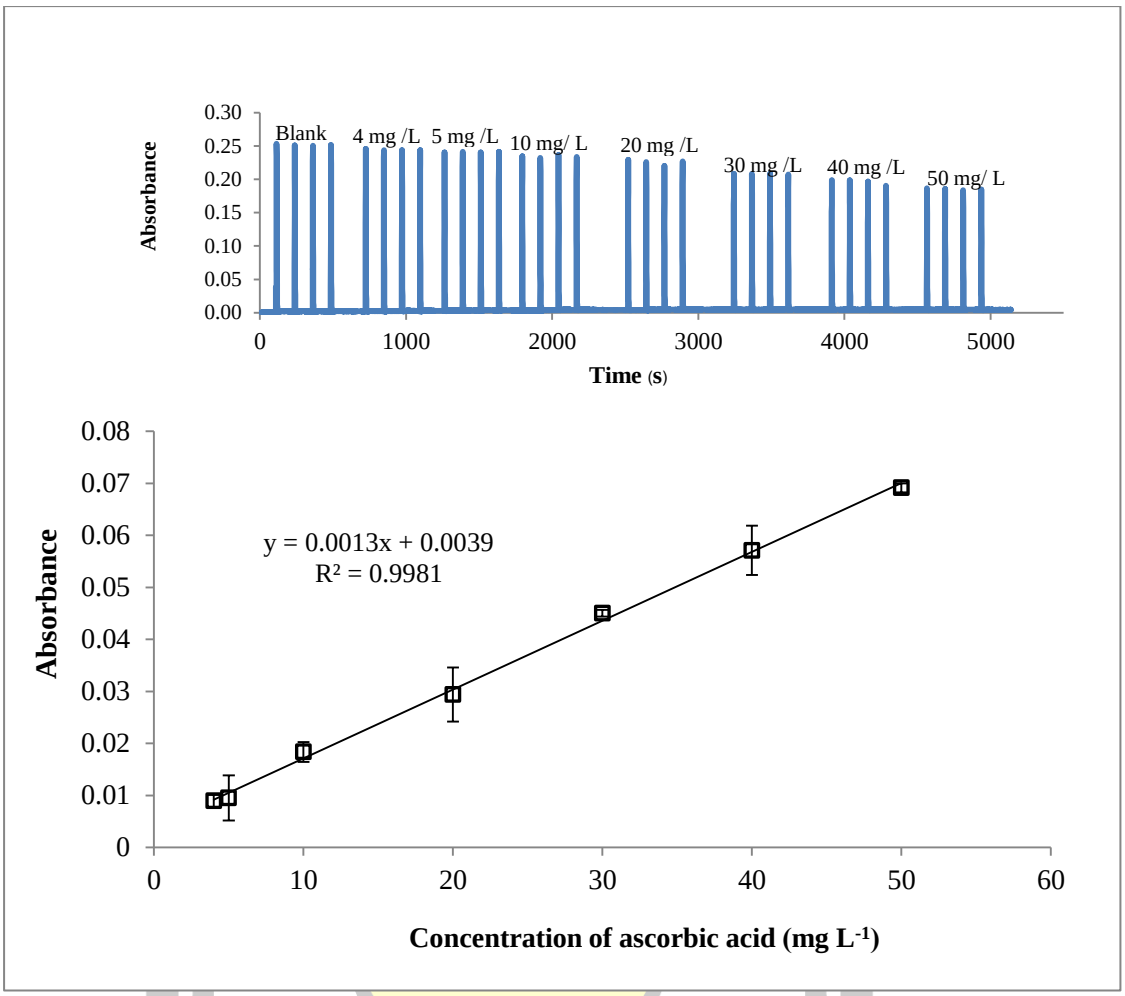


Figure 35 Calibration curve of ascorbic acid at 565 nm using the proposed method

Table 50 Analytical performance for determination of iron(III) by developed method

Linear range (mg L^{-1})	4-50
Linear equation	y=0.0013x+0.0039
R^2	0.9981
Limit of detection (mg L^{-1})	1.20
Limit of quantification (mg L^{-1})	4.00
Sample rate (h^{-1})	17

4.7 Applications

4.7.1 Application of SI system developed method for the iron determination

The developed method was applied to determine of iron(III)in water, rice and indigenous vegetable samples. The results are summarized in Table 51, 52 for water, rice and vegetable samples, respectively. The concentration of iron(III) was found in water sample number 3, 4, 9 and 10 in the range 0.38-1.13 mg L^{-1} which iron content was provided over maximum concentration limit set by WHO. Iron was found between 8.26-101.61 mg kg^{-1} and 57.07-334.07 mg kg^{-1} for rice and vegetable samples, respectively. High contamination of iron was observed in vegetable more than rice sample. Results obtained from the developed method were compared to the standard method by using FAAS. According to pair *t-test* at 95% confidence level, the results obtained from both the method are no statistical difference (t_{cal} =1.44, t_{table}=2.26), (t_{cal} = 0.58, t_{table} = 2.37) and (t_{cal} = 0.09, t_{table} = 2.57) for water, rice and vegetable samples, respectively. Furthermore, the accuracy as the percentage recovery was investigated by adding standard iron(III) at 3, 5, 7 mg L^{-1} for water samples and 2, 4 mg L^{-1} for rice and vegetable samples, respectively which were determined by the proposed method. The results of the percentage recoveries of iron in the ranging were obtained from 95.15-103.75%, 83.06-99.05% and 81.20-96.52% for water, rice and vegetable samples, respectively. It can be concluded that, this method was provided rapid, selective, sensitive, low waste production, low chemical consumption, cost-effective and environmentally friendly method for the determination of iron content in water, rice and vegetable samples. Moreover, this approach is that used a non-toxic reagent unlike the some other methods.

Table 51 Iron contents in water samples obtained by SI spectrophotometric and FAAS methods (*n=3*)

Water samples	Iron (mg L^{-1})		Mean recovery	Iron in water (mg L^{-1})	
	Added	Found		Proposed metho	FAAS
W1	1	1.006±0.002	99.55	ND	ND
	3	3.029±0.001	100.96		
	5	5.023±0.002	100.47		
W2	1	1.000±0.002	100.04	ND	ND
	3	3.006±0.003	100.20		
	5	4.994±0.001	99.88		
W3	1	0.990±0.001	99.02	1.133±0.001	1.189±0.0 02
	3	2.998±0.003	99.95		
	5	5.044±0.002	100.88		
W4	1	0.995±0.009	99.59	0.537±0.002	0.531±0.005
	3	3.002±0.001	100.107		
	5	5.111±0.002	102.21		
W5	1	1.002±0.003	97.75	ND	ND
	3	3.011±0.005	99.76		
	5	5.003±0.002	99.65		
W6	1	1.009±0.002	100.96	ND	ND
	3	2.854±0.002	95.15		
	5	4.948±0.003	98.96		
W7	1	1.009±0.002	100.93	ND	ND
	3	3.001±0.002	100.04		
	5	5.016±0.001	100.34		
W8	1	1.037±0.002	100.02	ND	ND
	3	3.021±0.008	99.46		
	5	4.961±0.002	99.22		
W9	1	1.007±0.008	100.70	0.384±0.002	0.383±0.003
	3	2.971±0.002	99.05		
	5	1.019±0.002	101.98		
W10	1	0.999±0.002	99.90	0.767±0.001	0.776±0.008
	3	3.084±0.003	102.83		
	5	5.187±0.002	103.75		

*ND is not detectable.

Table 52 Iron contents in rice and vegetable samples obtained by SI spectrophotometric and FAAS methods (*n=3*)

Samples		Iron (mg L^{-1})		Mean recovery		Iron content (mg kg^{-1})	
	Added	Found					
		Proposed method	FAAS	Proposed method	FAAS	Proposed method	FAAS
Rice samples							
Sinlek 1	2	1.795±0.028	1.726±0.002	89.61	86.00	9.795±0.041	9.756±0.003
	4	3.924±0.015	3.814±0.004	98.07	95.15		
Sinlek 2	2	1.832±0.040	1.623±0.003	91.92	81.20	101.027±0.010	100.729±0.001
	4	3.961±0.033	3.824±0.007	99.05	95.58		
Sinlek 3	2	1.970±0.010	1.901±0.003	98.40	94.90	52.507±0.018	50.278±0.002
	4	3.662±0.036	3.371±0.007	91.46	84.53		
Sinlek 4	2	1.913±0.039	1.803±0.003	95.57	89.80	8.571±0.015	8.725±0.003
	4	3.884±0.033	3.371±0.007	97.04	84.18		
Sinlek 5	2	1.701±0.013	1.931±0.020	85.00	96.52	12.438±0.014	13.392±0.003
	4	3.341±0.023	3.340±0.020	83.59	83.40		
Brown rice	2	1.792±0.011	1.760±0.001	89.53	88.00	ND	ND
	4	3.322±0.010	3.600±0.008	83.06	90.00		
Hhom-nin rice1	2	1.931±0.020	1.955±0.002	96.54	97.65	9.958±0.038	10.404±0.003
	4	3.642±0.024	3.212±0.010	90.06	80.25		
Hhom-nin rice2	2	1.935±0.010	1.940±0.010	96.51	98.85	17.503±0.017	17.404±0.009
	4	3.544±0.023	3.273±0.011	88.55	81.83		
Vegetable samples							
Kale	2	1.848±0.025	1.840±0.005	92.42	92.52	87.254±0.011	87.740±0.016
	4	3.762±0.027	3.780±0.015	94.16	94.50		
Wildbetal leafbush	2	1.799±0.026	1.839±0.010	89.97	92.00	123.555±0.010	132.880±0.066
	4	3.759±0.001	3.540±0.022	93.97	88.48		
Bamboo grass	2	1.672±0.039	1.687±0.005	83.47	84.15	223.347±0.029	233.554±0.099
	4	3.515±0.025	3.683±0.015	87.87	92.00		
Thai copper pod	2	1.837±0.005	1.974±0.089	91.85	98.65	334.071±0.010	311.031±1.803
	4	3.263±0.020	3.681±0.038	81.54	92.10		
Sweet basil	2	1.947±0.023	1.871±0.018	97.48	93.60	57.066±0.028	62.028±0.242
	4	3.918±0.036	3.822±0.038	97.98	95.50		
Siamese neem tree	2	1.891±0.037	1.773±0.010	95.00	88.50	122.048±0.020	117.067±0.051
	4	3.836±0.027	3.571±0.181	95.92	89.25		

*ND is not detectable.

4.7.2 Application of developed method for ascorbic acid quantification

The results of ascorbic acid concentration in pharmaceutical samples using the developed method compared with concentration of ascorbic acid as shown on the label (Label amount) was presented in Table 53. It was found that the ascorbic acid content in the pharmaceutical samples was not differed from the label values . The percentage label was 95.91-100.01.

Table 53 Determination of ascorbic acid in pharmaceutical samples

Sample	Ascorbic acid content			%Label [C]
	Stated [a] (mg)	Titration method [b]	Proposed method [b]	
S1	25	25.36±0.15	24.04±0.46	96.14
S2	25	25.45±0.21	24.93±0.68	99.71
S3	25	25.00±0.17	24.44±0.23	97.75
S4	25	24.94±0.10	25.00±0.23	100.01
S5	30	28.30±0.15	28.77±0.65	95.91

[a] Values for samples in mg per tablet, sachet or dose
[b] Titration method n=3, proposed method n=3
[C] Percentage label

Comparison the results of ascorbic acid concentration in the pharmaceutical using the developed method with the titration standard method, it was found that the results of the two methods were not significantly different at 95% confidence level (t_{cal} = 1.23, t_{crit} = 2.78).

4.8 Interference study

4.8.1 Effect of interference for the iron(III) determination

The effect of some interference species on the determination of iron(III) was investigated. Various concentrations of species ion were spiked into a standard solution of 0.5 mg L^{-1} iron(III). The tolerance limit is defined as the concentration of interference species causing absorbance error ±5% for the determination of iron(III). Tolerance limit of some interference ion show in Table 54.

Table 54 Interferences for the determination of iron(III)

Interference ions	Concentration ratio of iron(III) : Metal ion (mg L^{-1})
Na^{+1}	0.5:10
SO_4^{2-} and Ni^{2+}	0.5:5
Al^{3+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, Mn^{2+}, Br^-, NO_2^-, Cl^-, CO_3^{2-}, and NO_3^-	0.5:3
Ca^{2+} and CH_3COO^-	0.5:2
Fe^{2+}	0.5:1.5

The most serious interferences were caused by Fe^{2+}. However, the dilution samples may be avoided interferences. Moreover, Fe^{2+} is the contestable formation of the complexes with betel nut leading to low absorption in the previous results. Therefore, no interferences can be considered in this case.

4.8.2 Effect of interference for the ascorbic acid determination

In order to the possible analytical application of spectrophotometric method is described, the influence of species that coexist on the determination of ascorbic acid in real samples were investigated on the determination of 10 mg L^{-1} ascorbic acid. The tolerance limit was taken as the amount of coexist compounds which caused an absorbance error of ±5% for determination of ascorbic acid. Tolerance limit of some interference ion show in Table 55.

Table 55 Interferences for the determination of ascorbic acid

Interference	Concentration ratio of ascorbic acid: organic compounds (mg L^{-1})
Sucrose	10:50
Critic acid	10:20

CHAPTER V

CONCLUSION

The development of sequential injection spectrophotometry for determination of iron(III) is depended on interaction between iron(III) and natural reagent from betel nut. The presence of iron(III) was provided a maximum wavelength at 565 nm.

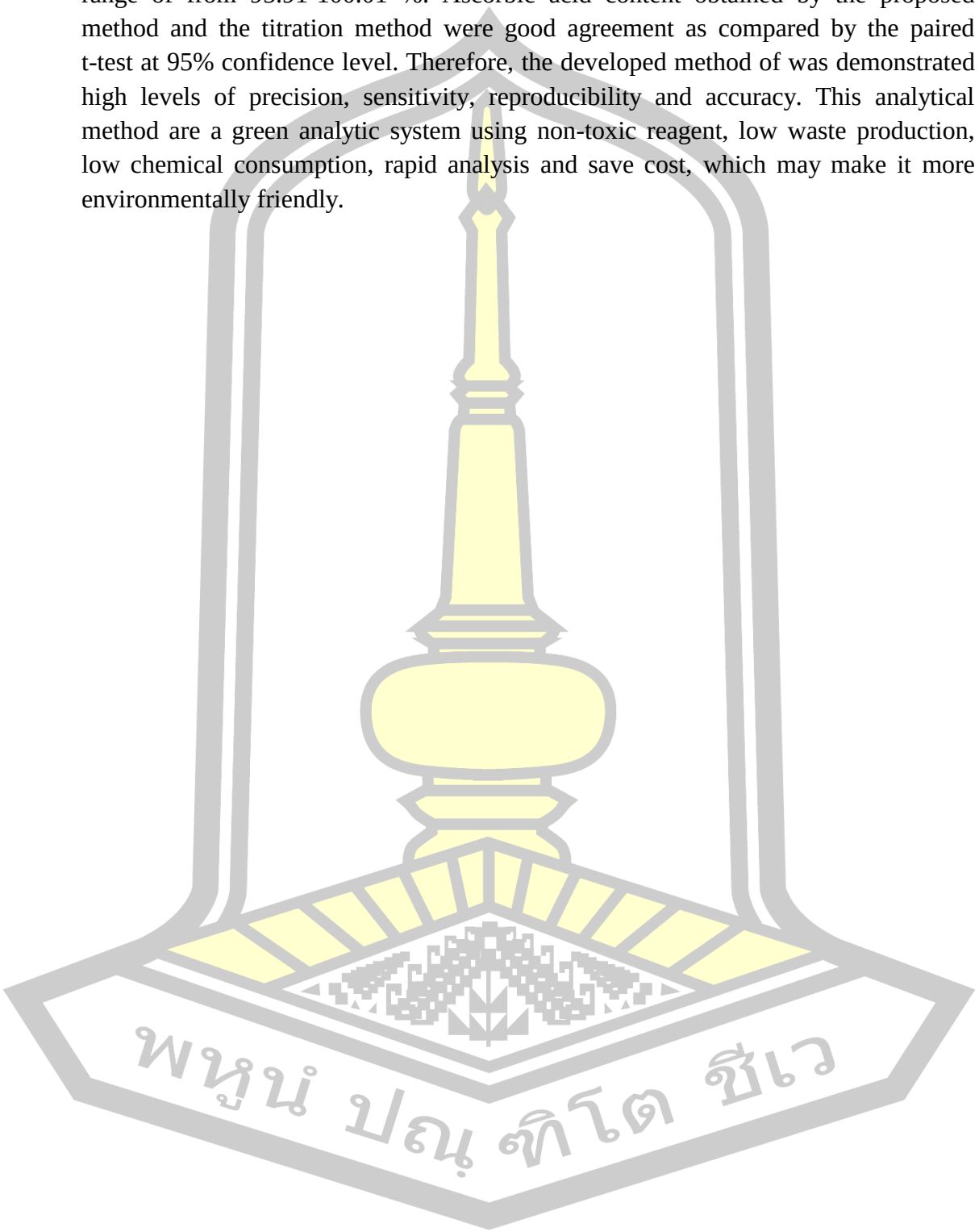
The betel nut extraction and the result color was changed to dark purple for was extracted using 0.5 g with hot water extraction for 20 minutes. The iron(III)-betel nut complex was stable at 0.7 mol L^{-1} acetate buffer pH 5.5. The optimum conditions for the determination of iron(III) employing SI system was operated as follows sequence order R-B/SD/R-B/SD/R-B/SD/R-B, mixing coil length 100 cm, flow rate 50 µL s^{-1} volume of standard 180 µL and volume of R-B 120 µL.

This developed method provided a linearity range for determination of iron(III) in range 0.2-10 mg L^{-1} with a regression equation: y=0.0141x-0.0023 and the correlation coefficient of 0.9995. The limit of detection (LOD) and limit of quantification (LOQ) were 0.06 and 0.20 mg L^{-1}, respectively. Relative standard deviations (%RSD) of four studied concentration levels for repeatability were 3.22, 3.37, 2.26 and 0.70 (n=10), respectively and the reproducibility were 4.84, 4.93, 4.88 and 3.78 respectively (n = 10, 6 days). In this research, an analysis method was developed to determine of iron(III) in water rice and vegetable samples. The accuracy of method presented as the percentage recovery were found in the range of 95.15-103.75%, 83.06-99.05% and 81.20-95.52% for water, rice and vegetable samples, respectively. Iron content obtained by the proposed method and the FAAS method were good agreement as compared by the paired t-test at 95% confidence level.

The optimum conditions for the determination of ascorbic acid by the proposed method was utilized sequence order R-B/Iron(III)/SD/R-B/SD/ Iron(III)/SD/R-B/SD/Iron(III)/R-B, concentration of iron(III) 40 mg L^{-1}, mixing coil length 100 cm, flow rate 70 µL s^{-1}, volume of standard 100 µL, volume of iron(III) 120 µL and volume of R-B 80 µL.

Determination of ascorbic acid by the developed method is based on the redox-reaction between ascorbic acid and iron(III) ions using natural reagent extracted from betel nut. The developed method provided a linearity range for determination of ascorbic acid in the range 4-50 mg L^{-1} with a regression equation: y=0.0013x+0.0039 and the correlation coefficient of 0.9981. The limit of detection (LOD) and limit of quantification (LOQ) were 1.20 and 4.00 mg L^{-1}, respectively. Relative standard deviations (%RSD) of two studied concentration levels for repeatability were 3.44 and 0.52 (n=10), respectively and the reproducibility were 4.72 and 1.39 respectively (n = 10, 5 days). In this research, an analysis method was developed to determine of

ascorbic acid in pharmaceutical samples. The percentage label was obtained in the range of from 95.91-100.01 %. Ascorbic acid content obtained by the proposed method and the titration method were good agreement as compared by the paired t-test at 95% confidence level. Therefore, the developed method of was demonstrated high levels of precision, sensitivity, reproducibility and accuracy. This analytical method are a green analytic system using non-toxic reagent, low waste production, low chemical consumption, rapid analysis and save cost, which may make it more environmentally friendly.



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