

## A GREEN COLORIMETRIC METHOD USING GUAVA LEAVES EXTRACT FOR QUALITY CONTROL OF IRON CONTENT IN PHARMACEUTICAL FORMULATIONS

(Kaedah Kalorimetrik Hijau Menggunakan Ekstrak Daun Jambu Bagi Kawalan Kualiti Kandungan Besi di dalam Formulasi Farmaseutikal)

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Received: 17 April 2019; Accepted: 22 June 2019

### Abstract

A cost-effective and environmentally friendly approach using a simple micro-volume colorimetric method with non-synthetic reagent from plant extracts has been proposed. The crude aqueous extracts from dried guava leaves were utilized as an alternative natural reagent for quantification of iron. The method was based on the measurement of a dark brown complex formed by the reaction between Fe(III) and the crude aqueous extracts in acetate buffer pH 4.5 at 562 nm. The optimum conditions for the extraction parameters such as type of solvent, extraction time and mass of dried plant were investigated. Under the optimum conditions, a linear calibration graph in the range of 1.0 - 10.0 mg L<sup>-1</sup> Fe(III) was obtained with limits of detection and quantification of 0.30 and 1.0 mg L<sup>-1</sup> Fe(III), respectively. Relative standard deviations of 2.6 and 2.1% were achieved for 2.0 and 4.0 mg L<sup>-1</sup> Fe(III) (n=7), respectively. The developed method was successfully applied to quality control of iron in antianemic drug samples. The results are in good agreement with those obtained by the FAAS method at the 95% confidence level. High percentage recoveries between 93 and 99% were obtained.

**Keywords:** guava leaf, natural reagent, colorimetry, iron, pharmaceutical formulations

### Abstrak

Pendekatan yang mesra alam dan kos efektif menggunakan kaedah kalorimetrik isipadu-mikro yang mudah dikemukakan dalam kajian ini. Ekstrak akues dari daun jambu yang dikeringkan telah digunakan sebagai alternatif reagen semulajadi bagi pengkuantitian kandungan besi. Kaedah berasaskan pengukuran kompleks coklat gelap yang terhasil melalui tindak balas Fe(III) dan bahan mentah ekstrak di dalam larutan penimbil asetat pH 4.5 pada 562 nm. Keadaan optimum untuk parameter pengekstrakan seperti jenis pelarut, masa pengekstrakan dan jisim daun yang dikeringkan telah dikaji. Pada keadaan optimum, graf kalibrasi linear diperolehi pada julat 1.0 - 10.0 mg L<sup>-1</sup> Fe(III) dengan had pengesanan dan pengkuantitian masing-masing ialah 0.30 and 1.0 mg L<sup>-1</sup> Fe(III). Sisihan piawai relative dicapai pada 2.6 dan 2.1% masing-masing bagi 2.0 and 4.0 mg L<sup>-1</sup> Fe(III) (n=7). Kaedah yang dibangunkan telah berjaya digunakan bagi tujuan kawalan kualiti besi di dalam sampel dadah antianemik. Keputusan yang diperolehi adalah standing dengan hasil yang diperolehi menggunakan kaedah FAAS pada aras keyakinan 95%. Peratus perolehan semula yang tinggi antara 93 dan 99% telah diperolehi.

**Keywords:** daun jambu, reagen semulajadi, kalorimetri, besi, formulasi farmaseutikal

### Introduction

Iron (Fe) is an essential mineral. Iron-deficiency anemia [1] is a serious health problem affecting a large proportion of the world's population especially high prevalence in women and children. Most iron-deficiency anemias respond well to treatment with oral or parenteral iron which contains iron (II) or iron(III) compounds to increase the number of red blood cells [2]. However, pharmaceuticals often cause side effects: nausea, anorexia, diarrhea and metal taste in the mouth [3]. Therefore, it is important to develop a simple, fast and inexpensive method for quality control of antianemic drugs and determination of iron content in pharmaceutical formulations, especially when a lot of samples have to be analyzed in the minimum of time.

Analytical techniques commonly used for iron determination in pharmaceutical formulations include flame atomic absorption spectrometry (FAAS) [4], inductively coupled plasma-optical emission spectrometry (ICP-OES) [5], and spectrophotometry using various complexing agents such as 4-(2-pyridylazo) resorcinol [6], pyrocatechol [7], ferrozine [8], Ferene S [8], 1,10-phenanthroline [8,9] and Tiron [10, 11].

Nowadays, green analytical technique or procedure which concerns the use of environmentally friendly chemicals and small-volume process is of much interest [12]. The extracts of plant or animal tissues with little purification or modification, natural reagents, have been used instead of high purity chemical reagents when a reagent is only required in excess, or a crude extract contains the necessary active compound [13]. The crude plant extracts including guava (*Psidium guajava* Linn.) leaves extract [14], green tea (*Camellia sinensis*) extract [15,16], *Phyllanthus emblica* Linn. [17] and *Smilax china* L. root extract [18] were reported as chromogenic agent for analysis of iron in combination with flow-based analytical techniques.

Guava (*Psidium guajava* L.) is widely cultivated and its fruit is popular in Asia. Literature studies considered these leaves as a promising source of phenolic compounds such as gallic acid, ferulic acid, catechin, quercetin and kaemferol [19-21]. Phenolic compounds contain a hydroxyl group are responsible for binding to or chelating with metal ions and phenolic groups in the presence of a third adjacent hydroxyl (pyrogallols) show increased stability with the Fe(III) ion [22].

In 2005, fresh guava leaves extract has been published as a model natural reagent for iron(III) analysis in spiked water samples using flow injection analysis system [14]. However, the low sensitivity was obtained and the reproducibility for extraction of fresh guava leaves extracts is low depending on the batch of fresh guava leaves and also on the season. Until now, the application of guava leaves extract for iron determination in real samples was not published elsewhere. Therefore, this work was aim to develop a simple and reliable method using natural reagent extracted from dried guava leaves as chromogenic reagent for iron determination in pharmaceutical samples. The microplate reader with visible detection was used to minimize the reagent/chemical usage, reduce the waste production during analysis, and provide fast measurement.

### Materials and Methods

#### Chemicals and reagents

All chemicals used were of analytical reagent grade. Deionized water from a Simplicity 185 (Millipore, Billerica, MA, USA) with resistivity of 18.2 M $\Omega$  cm was used throughout the experiments. A 1000 mg L<sup>-1</sup> Fe(III) standard solution for FAAS (Merck, Darmstadt, Germany) was used for all experimental study. Working standard solutions of iron with different concentrations were prepared by appropriately diluting the stock solution. Acetate buffer solutions at different pH were prepared from sodium acetate (Carlo Erba, Milano, Italy) and acetic acid (Carlo Erba, Milano, Italy). Methanol (Merck, Darmstadt, Germany), ethanol (Merck, Darmstadt, Germany), and acetone (Carlo Erba, Milano, Italy) were used as extracting solvent. Hydrochloric acid (HCl, 37%) (Univar, Ingleburn, New South Wales, Australia) was used for dissolution of the samples. Tannic acid (Carlo Erba, Milano, Italy), Folin-Ciocalteu reagent (Merck, Darmstadt, Germany), and sodium bicarbonate (Carlo Erba, Milano, Italy) were used for analysis of total phenolic contents.

### **Instrumentation**

A visible spectrophotometer, Genesys™ 20 (Thermo Fisher Scientific, USA) with Thermo Scientific VISIONlite 5 software was used for scanning the absorbance at selected wavelengths. A microplate reader, EZ Read 800 (Biochrom, UK) was used for micro-volume colorimetric determination using 96 well microplate. All pH measurements were conducted using a pH meter (Model 713, Metrohm, Switzerland). The FAAS measurements were performed with an Agilent 280FS AA atomic absorption spectrometer (Agilent Technologies, CA, USA) equipped with a deuterium background corrector. Detection wavelength for Fe was 248.3 nm and all instrumental conditions were followed according to the manufacturer's recommendation using an air/acetylene flame.

### **Preparation of natural reagent**

Guava leaves (> 5 kg) were collected in August 2017 and then cut into small pieces, cleaned, washed with deionized water and air dried. The dried leaves were ground to fine powder form using a blender machine (La Moulinette DPA 130, Tefal, France) and then homogeneously mixed and stored in a dry place until required for use.

Plant powder (5.0 g) and deionized water or other solvents (100 mL) were stirred in an extraction flask for 30 minutes. The extract was filtered through a filter paper (Whatman® No.4, UK) and made up to a volume of 100 mL with deionized water.

### **Total phenolic contents**

Total phenolic contents, in terms of tannic acid equivalents of the guava leaf extracts, were determined using the Folin-Ciocalteu reagent assay (method 952.03) [23]. An aliquot of guava leaves extract or standard tannic acid solution was pipetted into a 100 mL volumetric flask containing 75 mL of water. Then, 5 mL of Folin-Ciocalteu reagent and 10 mL of sodium carbonate saturated solution were added and the volume was adjusted with deionized water. The solutions were mixed and allowed to stand for 30 minutes at room temperature. Finally, absorbance was measured at 760 nm.

### **Sample preparation**

Ten commercially available pharmaceutical samples of two different forms of ferrous fumarate (F1-F7) and ferrous sulfate (S1-S3) were selected and bought directly from drug stores in Maha Sarakham Province, Thailand. Prior to analysis, a set of 20 tablets was weighed and manually ground to fine powder with an agate mortar and pestle.

Samples were prepared following the Association of Official Analytical Chemists (AOAC) method 977.30 [24]. Briefly, a portion of fine powder containing about 60 mg Fe, was accurately weighed and mixed with 100 mL of water and 4 mL of concentrated HCl. The mixture was boiled in a steam bath for 30 minutes and then allowed to cool to room temperature before dilution with water to a volume of 200 mL, placed in a volumetric flask and filtrated through a filter paper (Whatman® No.1, UK). Iron content was analyzed by FAAS. For analysis by the proposed colorimetric method, hydrogen peroxide was added to ensure the complete oxidation of Fe(II) to Fe(III).

### **Micro-volume colorimetric assay**

This assay was performed in 96 well microplate and reactive solutions were measured by a micro-volume colorimeter. For the construction of standard curves, 50 µL of natural reagent and 50 µL of acetate buffer pH 4.5 were added to each well of microplate. Then, aliquots comprising seven different concentrations (0, 1, 2, 4, 6, 8, and 10 mg L<sup>-1</sup>) of iron standard solutions or sample solutions were added. Deionized water was then used to make up the volume to 250 µL per well. The microplate was shaken to mix the contents, followed by reading at 562 nm using a microplate reader.

## **Results and Discussion**

### **Extraction of natural reagent from guava leaves**

The main components in guava leaf are phenolic compounds [19-21]. Type of solvent has a strong impact on the yield of extraction. In this study, different polar solvents such as methanol, ethanol, acetone and water were investigated. Total phenolic contents (as tannic acid) of each extract were evaluated. The methanol extract gave the highest tannic acid (3.19 g/100g). The total phenolic contents (as tannic acid) for ethanol, acetone and water were 1.98, 0.37 and 2.85 g/100g, respectively. However, it would be more beneficial if the extraction could be carried out

in the aqueous phase and used directly without purification [13]. Therefore, water was selected as the suitable extraction solvent for its simplicity and the reduced of amounts of chemicals used.

One interesting property of polyphenolic compounds is their ability to influence iron absorption. For the preliminary study, a simple extraction method for guava leaves was performed by stirring with water. Crude aqueous extract from guava leaf was used as natural reagent without purification. Complexation of crude extract with iron and other metal ions was subjected to preliminary investigation by the batchwise method. As shown in Figure 1, the mixture of crude aqueous extracts with a buffer solution at 4.5 had a yellow color. After mixing the extracts with other metal ions such as copper, aluminium and iron, marked changes in color were observed only for iron which turned from yellow to dark brown. Obvious differences in absorption spectra of natural reagent and natural reagent with iron were observed with maximum absorption wavelength of 570 nm. This result indicated the possibility of iron complexation with crude extract from guava leaves.

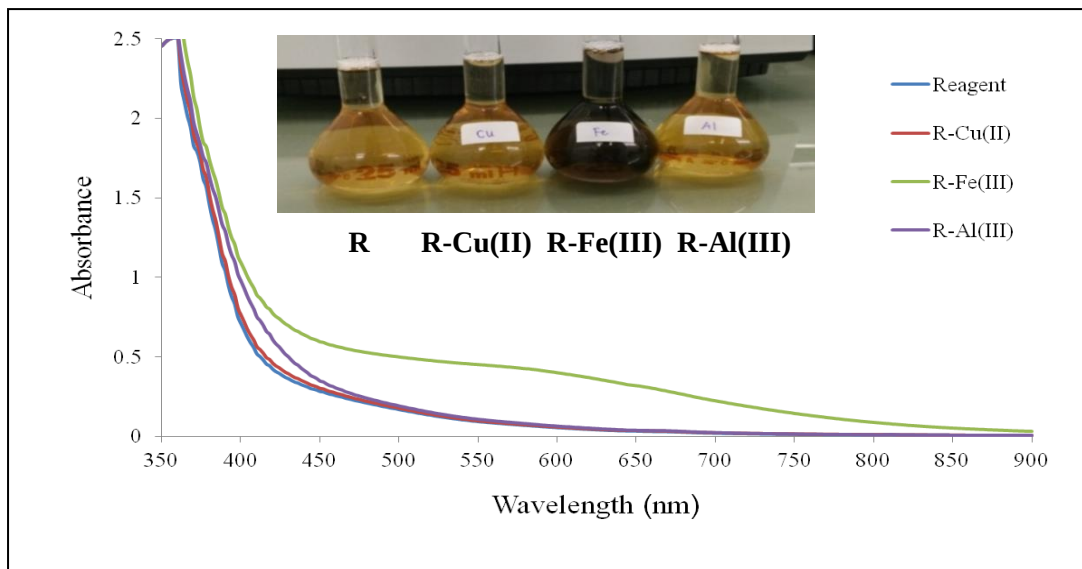


Figure 1. Visual detection and visible spectra of natural reagent (R) with Cu(II), Fe(III) and Al(III) at pH 4.5

Effect of pH on the complexation of natural reagent with iron was investigated over the range of 3.5 – 6.0 using 1 M acetate buffer ( $n=3$ ). As shown in Figure 2, absorbance of iron-reagent complex increased with pH from 3.5 to 4.5. At pH 5.0 – 6.0, the turbid solutions were observed, thus, the absorbance cannot be measured. This result indicated that the iron-reagent complex forms efficiently in the range of pH 4.5. Therefore, the acetate buffer pH 4.5 was chosen.

Interaction of Fe(II)/Fe(III) with natural reagent extract was also investigated and compared for sensitivity. Both Fe(II) and Fe(III) formed color complexes with guava leaves extract and Fe(III)-natural reagent complex had higher molar absorptivity. Therefore, to obtain the best sensitivity, Fe(III) was chosen as the standard to form complexes with natural reagents for construction of calibration/standard addition graphs.

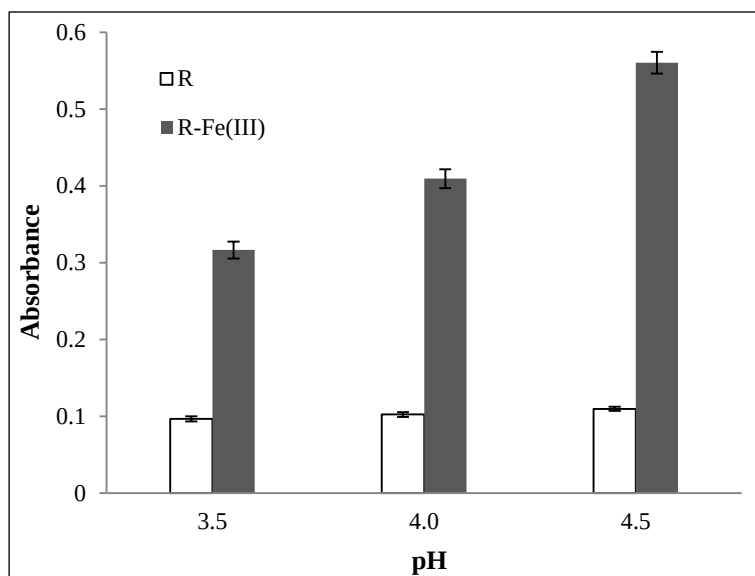


Figure 2. Effect of pH on the complexation of natural reagent with iron

The influence of extraction time on the extraction yield of phenolic compounds was investigated from 15, 30, 45 and 60 minutes (n=3). As shown in Figure 3, absorbance of the reagent and iron-reagent complex increased slightly with increasing extraction time from 15 to 30 minutes, and then remained constant with increasing extraction time from 30 to 60 minutes. This might be due to the excess total phenolic compounds in the extracts. Therefore, an extraction time of 30 minutes was selected as suitable.

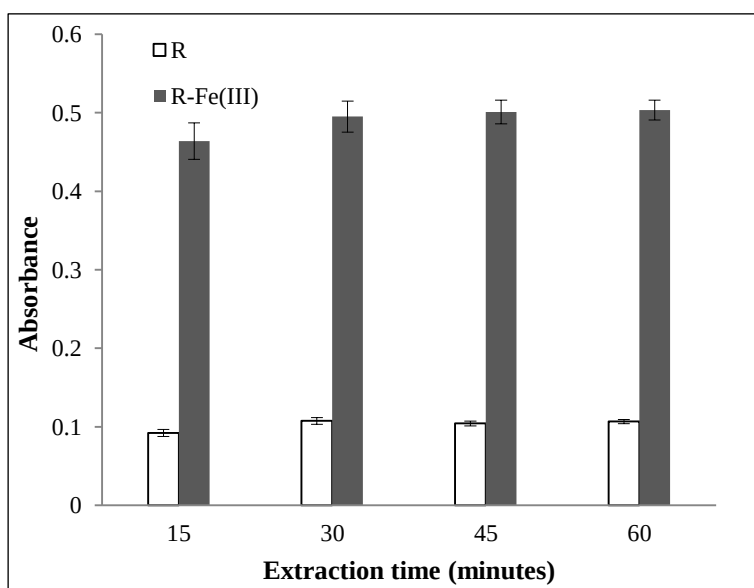


Figure 3. Effect of extraction time on the extraction yield of phenolic compounds

The amount of plant material related to the extraction yield was investigated by varying the weight of guava leaf powder from 3.0, 4.0, 5.0, and 6.0 g and extracting with 100 mL of deionized water (n=3). As shown in Figure 4,

the absorbance increased with increasing amounts of plant powder from 3.0 - 4.0 g, and then remained constant. The ratio of 5.0 g of plant material per 100 mL of water was selected for the extraction procedure.

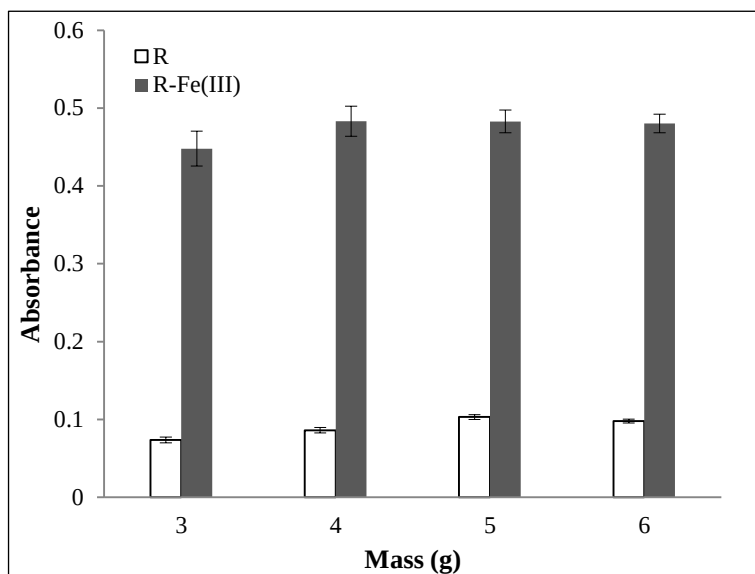


Figure 4. Effect of guava leaves mass on the extraction yield of phenolic compounds

To ensure the reproducibility of the various batches of natural reagent extracts for future use, the precision of absorbance of 10 batches of the extracts prepared independently was evaluated. The relative standard deviation (%RSD) for all 10 batches was 5.7% which is an acceptable range.

The stability of natural reagent and its complex were investigated. Absorbance of natural reagent with Fe(III) was recorded. A mixture of iron and freshly prepared extract was used for comparison. The signal of natural reagent did not show any loss in performance within 72 hours (3 days).

#### Analytical characteristics of the proposed micro-volume colorimetric method for iron

Analytical characteristics of the proposed colorimetric method were investigated using a microplate reader colorimeter at 562 nm. Under optimum extraction conditions as described above, standard calibration in the range of 1.0-10.0 mg L<sup>-1</sup> was constructed by plotting absorbance against iron concentration. A linear calibration graph was obtained with calibration equation  $y = 0.0527x + 0.1196$ ,  $R^2 = 0.9974$ . The limits of detection ( $3\sigma/s$ ) and quantification ( $10\sigma/s$ ) (where  $\sigma$  is the standard deviation of reagent blank ( $n=11$ ) and  $s$  is the slope of the calibration curve) for iron were obtained at 0.30 and 1.0 mg L<sup>-1</sup>, respectively. The relative standard deviation for seven replicate determinations of 2 mg L<sup>-1</sup> and 4 mg L<sup>-1</sup> were 2.6 and 2.1%, respectively.

The analytical performance of the proposed method with previous flow-based methods using other natural reagents such as fresh guava (*Psidium guajava* L.) leaves extract [14], green tea (*Camellia sinensis*) extract [15] *Phyllanthus emblica* L. extract [17] and *Smilax china* L. root extract [18] was summarized in Table 1. Although the proposed method was not as sensitive as some reported for other flow-based system, the sensitivity of this proposed method is enough to measure iron in pharmaceutical samples. The sampling rate of this method is comparable to those of previous flow-based system from its speed of simultaneously detection in 96 well of each microplate. Moreover, this method reduces contamination risk, performs on common and inexpensive equipment with a minimum of training. As compared to the fresh guava leaves extracts [14], the guava leaves extracts prepared from dried guava leaves powder could be a better alternative source of reagent because the dried plant powder can be stored for long time and its availability with homogeneity.

Table 1. The analytical characteristics of the colorimetric determination method for iron(III)

| Method               | Natural reagent                                         | Linear Range<br>(mg L <sup>-1</sup> ) | LOD<br>(mg L <sup>-1</sup> ) | Sampling<br>Rate<br>(h <sup>-1</sup> ) | Sample<br>Volume<br>(μL) | Ref.      |
|----------------------|---------------------------------------------------------|---------------------------------------|------------------------------|----------------------------------------|--------------------------|-----------|
| Flow injection       | Fresh guava ( <i>Psidium guajava</i> L.) leaves extract | 1 – 10                                | 1                            | 20                                     | 80 <sup>a</sup>          | [14]      |
| Flow injection       | Green tea ( <i>Camellia sinesis</i> ) extract           | 1.0 – 20.0                            | 0.05                         | 180                                    | 80 <sup>a</sup>          | [15]      |
| Flow injection       | <i>Phyllanthus emblica</i> L. extract                   | 0.50 – 20.0                           | 0.31                         | 90                                     | 200 <sup>a</sup>         | [17]      |
| Sequential injection | <i>Smilax china</i> L. root extract                     | 1 - 8                                 | 0.05                         | 12                                     | 150 <sup>b</sup>         | [18]      |
| Microplate reader    | Dried guava ( <i>Psidium guajava</i> L.) leaves extract | 1.0 – 10.0                            | 0.30                         | >90                                    | 250 <sup>b</sup>         | This work |

<sup>a</sup> sample volume only

<sup>b</sup> total analysis volume (sample+reagent+other solutions)

Effect of interfering species on the iron-natural reagent complex was investigated using the proposed method under optimum conditions. Various concentrations of foreign ions (Ca<sup>2+</sup>, Mg<sup>2+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup>, Cu<sup>2+</sup>, Mn<sup>2+</sup> and Cr<sup>3+</sup>) were spiked into a standard solution of 5 mg L<sup>-1</sup> Fe(III). An interfering concentration was considered as the value that caused signal variations higher than ±10%. Results showed that the system could tolerate spiked cations up to at least a 1:1 concentration ratio of iron to interfering cations. Higher interference ratios were not studied since these minerals are normally found in pharmaceutical formulations at lower amounts than iron compounds.

#### Application to pharmaceutical samples

The proposed method was employed for the determination of iron in pharmaceutical formulations in two different forms (ferrous fumarate and ferrous sulfate). Amounts of iron found in pharmaceutical samples after dissolution by standard method for analysis of iron in drugs [24] were compared with their label values and analyzed by FAAS. Results are presented in Table 2. Iron contents in all samples obtained from FAAS and proposed methods were determined in an acceptable range (90.0 – 110.0 %) of the labeled amounts. A *t*-test at 95% confidence limit, indicated that results obtained from both methods were in good agreement (*t*<sub>crit</sub> = 2.26, *t*<sub>cal</sub> = 1.07). Satisfactory recoveries in the range of 93-99% for all pharmaceutical samples were obtained. These results indicated that the proposed method was comparable to the reference method. This proposed method provides some advantages over the FAAS method including ease of operation, low cost of instrument, low chemical consumption, and high sample throughput.

Table 2. Determination of iron in pharmaceutical formulations (n = 3)

| Sample <sup>a</sup> | Label Amount<br>(mg Fe(II) tablet <sup>-1</sup> ) | Reference Method                           |                      | Proposed Method                            |                      |
|---------------------|---------------------------------------------------|--------------------------------------------|----------------------|--------------------------------------------|----------------------|
|                     |                                                   | Found<br>(mg Fe(II) tablet <sup>-1</sup> ) | % Label <sup>b</sup> | Found<br>(mg Fe(II) tablet <sup>-1</sup> ) | % Label <sup>b</sup> |
| F1                  | 200                                               | 188 ± 2                                    | 94                   | 198±3                                      | 99                   |
| F2                  | 200                                               | 196 ± 7                                    | 98                   | 201±11                                     | 101                  |
| F3                  | 200                                               | 191 ± 7                                    | 96                   | 203±1                                      | 102                  |
| F4                  | 200                                               | 196 ± 2                                    | 98                   | 198±1                                      | 100                  |
| F5                  | 200                                               | 194 ± 2                                    | 97                   | 195±2                                      | 98                   |
| F6                  | 149                                               | 153 ± 2                                    | 103                  | 156±2                                      | 105                  |
| F7                  | 200                                               | 219 ± 4                                    | 110                  | 213±2                                      | 106                  |
| S1                  | 135                                               | 121 ± 2                                    | 90                   | 122±3                                      | 90                   |
| S2                  | 200                                               | 182 ± 6                                    | 91                   | 180±7                                      | 90                   |
| S3                  | 200                                               | 184 ± 5                                    | 92                   | 188±7                                      | 94                   |

<sup>a</sup>F1-F7 : ferrous fumarate, S1-S3 : ferrous sulfate

<sup>b</sup>% Label = [found / label value] x 100

### Conclusion

A green, simple and reliable method based on natural reagent extracted from plant and micro-volume colorimetry at 562 nm was proposed for determination of iron. The crude aqueous extracts from the dried guava leaves can replace toxic chemical reagents for the quantitative analysis of iron content in pharmaceutical samples. The extraction procedure of dried guava leaves is simple and precise. One batch extraction of this natural reagent can be used for at least 3 days. The developed method provides low chemical usage, low cost of operation and high sample throughput. The satisfactory recoveries proved that the proposed method has high potential as a good alternative method for quality assurance of iron in pharmaceutical industry and low resource areas. In addition, the visual detection of this green approach trends to further development as a screening method for iron in ground water samples, which could be useful for developing countries.

### Acknowledgement

This study was financially supported by the Faculty of Science, Mahasarakham University. The authors gratefully acknowledge TRF Distinguished Research Professor Award Grant (DPG608002) (K. Gaudian), the Mahasarakham University Development Fund and the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Office of the Higher Education Commission, Ministry of Education. Additional support was received from the Center of Excellence for Innovation in Analytical Science and Technology (I-ANALY-ST), Chiang Mai University which was greatly appreciated. The authors wish to thank Ms. Pimpaka and Ms. Yonlada Karun for their invaluable assistance.

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