CHAPTER 3

RESULTS AND DISCUSSION

3.1 Optimization of flow injection amperometric method using triiodide as a reagent

In this work, flow injection amperormetric using triiodide ion as a reagent was investigated for estimation of antioxidative activtity. Triiodide ion was produced from the reaction of iodate with iodide in an acidic medium. Triiodide undergoes electrochemical reduction on a glassy carbon working electrode at 200 mV versus Ag/AgCl reference electrode producing electrical current. Antioxidant reacts with triiodide leading to the decrease in the electrical current which was directly proportional to antioxidative activity. The process was taking place following the equation 3.1.

$$IO_3^- + 5I^- + 6H^+ \rightarrow 3I_2 + 3H_2O$$

$$3I_2 + 3I^- \rightleftarrows 3I_3^-$$

$$3I_3^- + 6e^- \rightleftarrows 9I^-$$

$$C_6H_8O_6 + I_3^- \rightarrow C_6H_6O_6 + 3I^- + 2H^+$$

$$or\ Antioxidant + I_3^- \rightarrow oxidized\ antioxidant + 3I^- \quad (3.1)$$

Some parameters were studied as follow.

3.1.1 Concentration of potassium iodate

The effect of potassium iodate concentration on sensitivity was investigated in the concentration range of 15 -75 ppm. By considering the slope of calibration graph which was plotted between peak height obtained and concentration of ascorbic acid solution, it was found that the sensitivity was improved by increasing concentration of iodate as illustrated in Figure 3.1 since too low concentration of potassium iodate is not enough to produce triiiodide that required to react with high content of antioxidant. At the high concentration of iodate, the sensitivity was also decreased because too much triiodide was produced when standard or sample is injected so the observed current change from the baseline was unnoticeable. A concentration of 25 ppm of potassium iodate was selected to be the optimum value as it gave high sensitivity and low background signal.

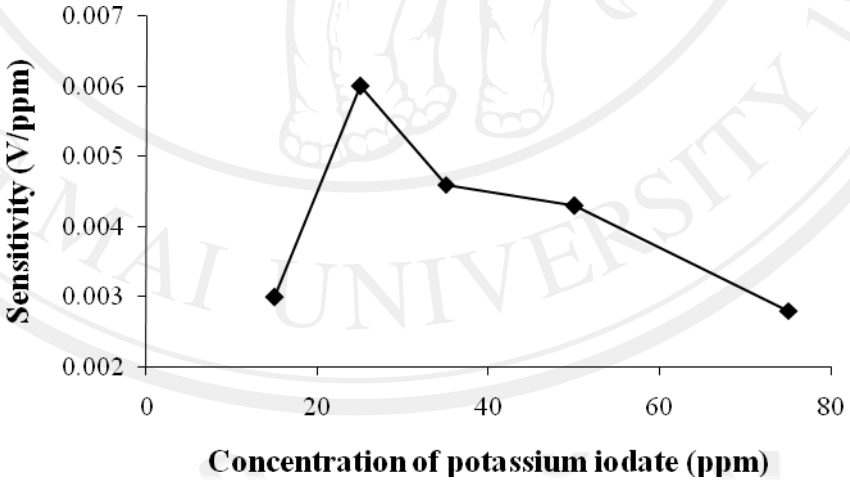

Figure 3.1 Effect of potassium iodate concentration on sensitivity of the method.

3.1.2 Concentration of potassium iodide

The effect of potassium iodate concentration on sensitivity was studied in the concentration range of 0.1 – 10 mM. Calibration graphs of standard ascorbic acid 8-100 ppm were constructed, and the sensitivity obtained from calibration graphs was used for comparisons. It was found that the sensitivity was improved by increasing iodide concentration as shown in Figure 3.2. In 0.1 mM potassium iodide too low concentration of triiiodide was resulted, hence low sensitivity was observed. But in the high concentration of potassium iodide, the sensitivity was also decreased because too high concentration of triiodide was produced, leading to small change of current from the baseline.

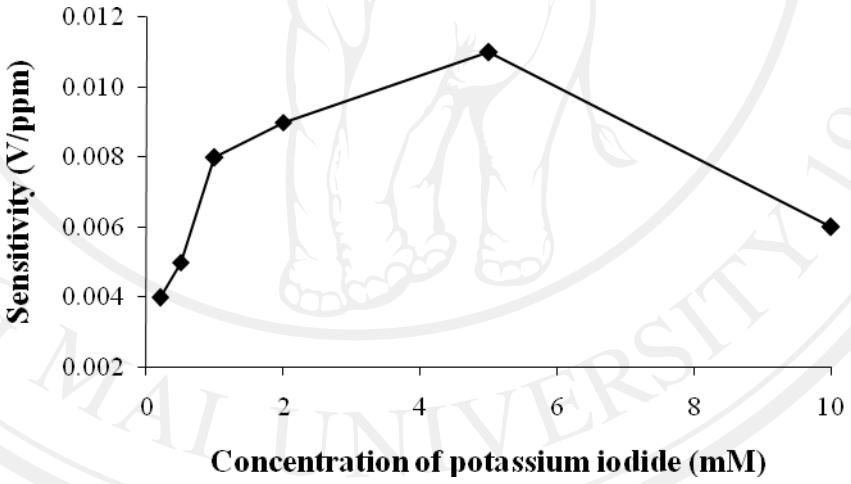

Figure 3.2 Effect of potassium iodide concentration on sensitivity of the method.

3.1.3 Concentration of hydrochloric acid

Effect of concentration of hydrochloric acid on sensitivity was studied in the range of 0.010 - 1.000 M. By considering the slope of calibration graph of standard ascorbic acid, it was found that the sensitivity was increasing when the concentration of

hydrochloric acid increased as illustrated in Figure 3.3, but in the very high concentration of hydrochloric acid, the sensitivity was decreased because high concentration of triiodide was produced when standard or sample was injected the observed signal was unchangeable.

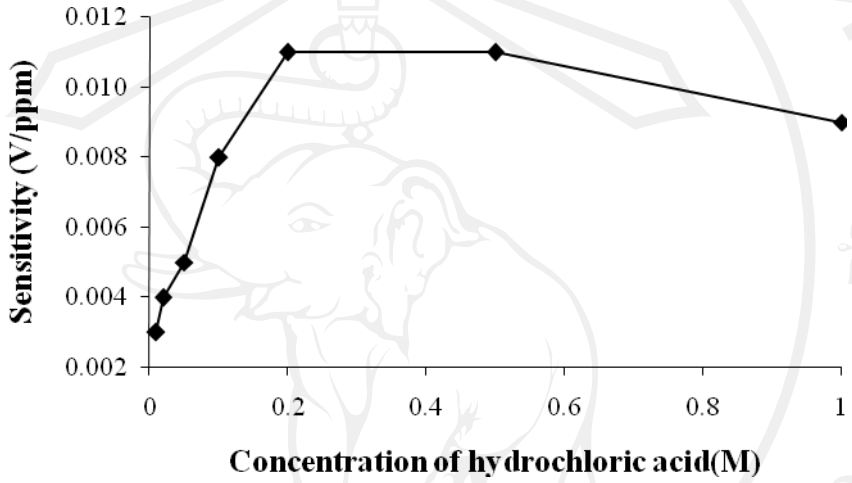

Figure 3.3 Effect of hydrochloric acid concentration on sensitivity of the method.

3.1.4 Total flow rate

Increasing the total flow rate is one of the effective ways to increase sensitivity of measurement for the fast reaction and it, reduce dispersion and increases sample throughput. However, the sensitivity usually decrease when too fast flow rate is used. Thus, the effect of total flow rate was optimized in the range of 2.0-4.5 ml.min^{-1}. The sensitivity obtained is shown in Figure 3.4. According to Figure 3.4, it was clearly observed that higher flow rate of the system (up to 4 ml.min^{-1}) led to an increase in sensitivity. Therefore, total flow rate of 4.0 ml.min^{-1} was chosen because it offered the highest sensitivity and gave high sample throughput.

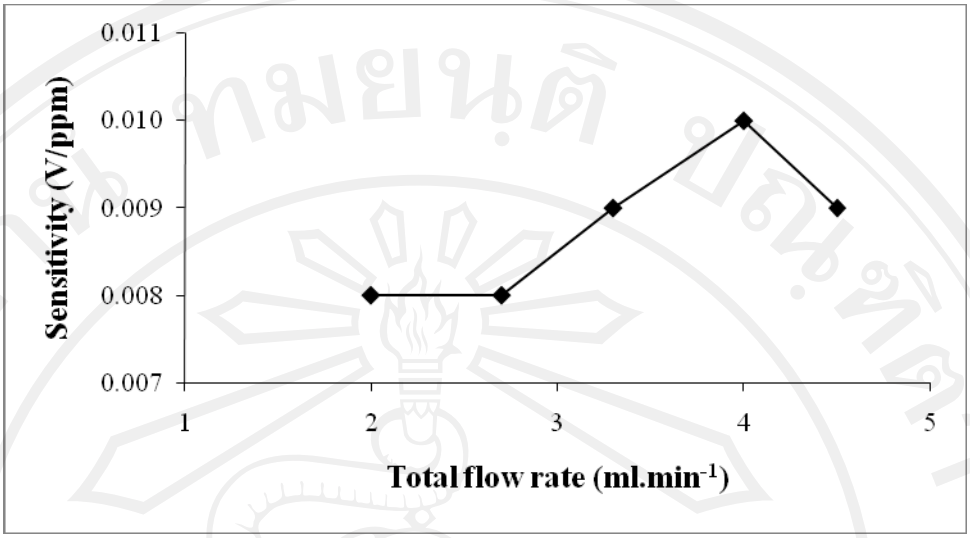

Figure 3.4 Effect of total flow rate on sensitivity of the method.

3.1.5 Summary of the selected conditions

The conditions for estimation of antioxidant activity using FI-amperometric method with triiodide as a reagent are summarized in the Table 3.1.

Table 3.1 The optimum condition of FI-amperometric method for estimation of antioxidant activity

Parameters	**Selected value**
Potassium iodate concentration	25 ppm
Potassium iodide concentration	5 mM
Hydrochloric acid concentration	0.2 M
Total flow rate	4.0 ml.min^{-1}
Sample volume	100 µL
Mixing coil	50 cm
Electrode	GCE
Electrode potential	200 mV versus Ag/AgCl

3.2 Analytical characteristics of the procedure

3.2.1 Calibration curves and limit of quantitative

Using the optimum condition in Table 3.1, the FIAgram and the calibration graph were obtained as shown in Figure 3.5 and Figure 3.6, respectively. The calibration graph is linear in the range of 8-100 ppm of ascorbic acid. The linear regression equation obtained was $y = 0.009x - 0.007$, $R^2 = 0.9990$ (where y and x are peak height obtained and ascorbic acid concentration, respectively). The limit of detection (LOD) is calculated from where S_a is the standard deviation of the y-intercept of the regression line and b is the slope of the calibration curve [6]. Limit of detection of 2 ppm was achieved.

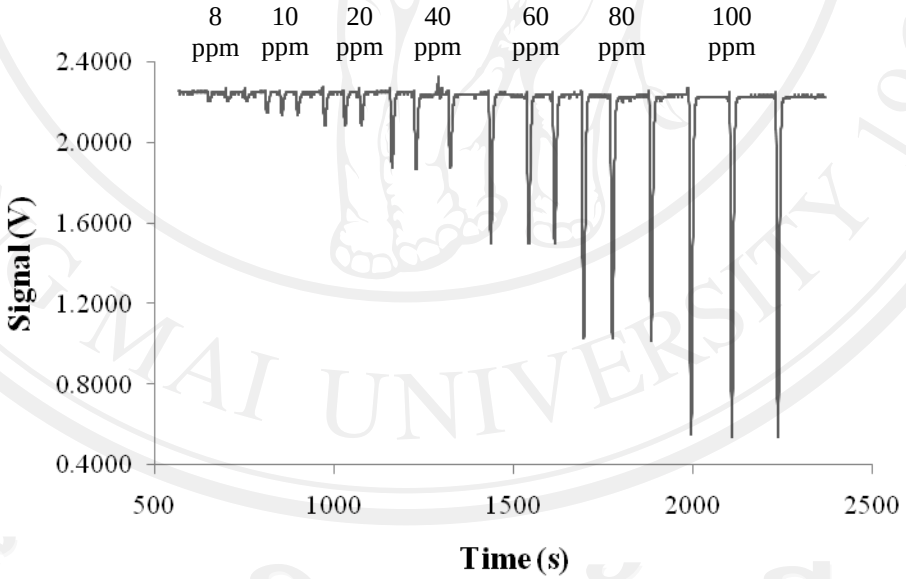

Figure 3.5 FIAgram for the estimation of antioxidant activity by FI-amperometric method.

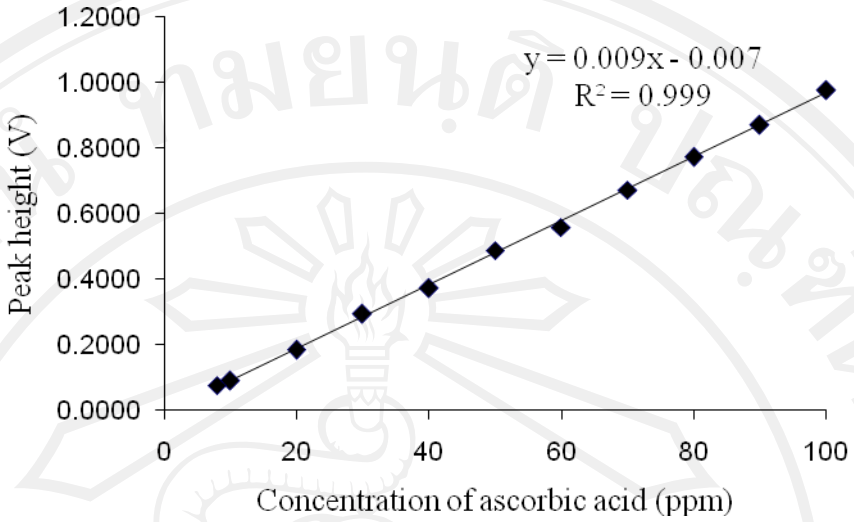

Figure 3.6 Calibration graph for the estimation of antioxidant activity by FI-amperometric method.

3.2.2 Precision study

3.3.2.1 Precision study of the method

The precision of the method was investigated by nine – replicate injections of ascorbic acid at low and high concentration level ca. 8 and 100 ppm. The percentage of relative standard deviation (%RSD) values used for evaluating the precision can be calculated from the equation 2.1 described in section 2.8.2. Results are given in Table 3.2. and it shows that the analytical system gives good reproducibility. The relative standard deviations were 1.2 and 1.6 for 8 ppm and 100 ppm, respectively.

Table 3.2 The precision of the system at two concentrations of ascorbic acid

Number of injection	Peak height (V)	
	8 ppm	100 ppm
1	0.0600	0.7200
2	0.0602	0.7358
3	0.0608	0.7477
4	0.0591	0.7285
5	0.0591	0.7264
6	0.0601	0.7455
7	0.0600	0.7480
8	0.0594	0.7224
9	0.0609	0.7206
Mean	0.0600	0.7328
SD	0.0007	0.0117
% RSD	1.2	1.6

3.2.2.2 Precision study of sample preparation method

Samples were prepared by weighing tea samples 1 g and boiled in 50 ml water for 30 min. Then, the solution was filtered through Whatman filter paper No. 1 and adjusted the volume of the filtrate to 50 ml in a volumetric flask. The precision of the sample extraction method was investigated by five – replicate extractions of three samples at low, medium and high content of antioxidant. Each extracted solution was then injected into the system in triplicate. The percentage of relative standard deviation (%RSD) values was used for evaluating the precision of the sample extraction method. It was calculated from the equation 2.1 and the results are given in Table 3.3. This sample preparation method had satisfactory reproducibility. The relative standard deviations were 7.07, 3.85 and 1.15 for sample 1, sample 2 and sample 3, respectively.

Table 3.3 The precision study of the sample preparation method

Number of sample prepration	Number of injection	Peak height (V)		
		Sample 1	Sample 2	Sample 3
1	1	0.4687	0.8374	0.8630
	2	0.4492	0.8297	0.8422
	3	0.4284	0.8204	0.8453
2	1	0.4109	0.8617	0.8478
	2	0.4111	0.8457	0.8415
	3	0.4131	0.8524	0.8462
3	1	0.4084	0.8325	0.8634
	2	0.4013	0.8353	0.8539
	3	0.4008	0.8306	0.8496
4	1	0.3693	0.7781	0.8435
	2	0.3684	0.7683	0.8320
	3	0.3608	0.7646	0.8315
5	1	0.4044	0.8125	0.8368
	2	0.4043	0.7906	0.8344
	3	0.3903	0.7842	0.8469
Mean		0.4060	0.8163	0.8452
SD		0.0287	0.0314	0.0097
% RSD		7.07	3.85	1.15

3.2.3 Recovery study

Recovery of the method was examined by spiking standard ascorbic acid solution into some selected samples. The recovery percentages were calculated from the results obtained from the calibration curve as compared to the expected values. The study on standard addition method indicates that sample matrix affected to the analysis result. With higher dilution (sample matrix decrease), the better percentage recovery was obtained. And from checking system which was examined by spiking standard ascorbic acid solution into standard ascorbic acid as sample solution. The recovery percentages were calculated similar to recovery of the sample. The percentages of recovery are close to 100% which indicated that the error did not come from the analytical system. The results are shown in Table 3.4 and 3.5. In Table 3.5,

the results obtained from the same sample but they have quite different value. Because the samples were stored for long before analysis so there may be some decomposed.

Table 3.4 The recovery percentages obtained by spiking ascorbic acid at 10-40 ppm into sample

Dilution Factor	sample	Spiking ascorbic acid (ppm)	Found signal(V)	Concentration found (ppm)	% Recovery
10	1	-	0.2241	13.58	-
		10	0.3555	19.83	84.12
		20	0.5245	27.88	83.04
		40	0.8649	44.09	82.29
10	2	-	0.7849	40.30	-
		10	0.8158	41.75	83.01
		20	0.8509	43.42	72.01
		40	1.025	51.72	64.41
10	3	-	0.8587	43.81	-
		10	0.8735	44.52	82.73
		20	0.9509	48.20	75.54
		40	1.100	55.30	65.98
12.5	1	-	0.0669	3.619	-
		10	0.1972	11.76	86.37
		20	0.3565	21.72	91.96
		40	0.6602	40.70	93.31
20	2	-	0.1054	6.025	-
		10	0.2333	14.02	87.48
		20	0.3617	22.04	84.70
		40	0.6511	40.13	87.19
20	3	-	0.1614	9.525	-
		10	0.2752	16.64	85.21
		20	0.4006	24.48	82.90
		40	0.6623	40.83	82.45
Checking system	1	-	0.2704	18.76	-
		10	0.4117	28.18	97.98
		20	0.5671	38.54	99.43
		40	0.8886	59.97	102.1
	2	-	0.3367	23.18	-
		10	0.5084	34.63	104.4
		20	0.6535	44.30	102.6
		40	0.9549	64.39	101.9
	3	-	0.4442	30.35	-
		10	0.5754	39.09	96.89
		20	0.7417	50.18	99.67
		40	1.060	71.43	101.5

Table 3.5 Comparisons content of antioxidant between calculated from standard addition graph and calibration graph

Dilution factor	Sample	Calibration graph mg/g $_{sample}$	Standard addition graph mg/g $_{sample}$
10	1	6.80	6.50
10	2	20.1	63.5
10	3	21.9	70.8
12.5	1	2.20	2.40
20	2	6.00	7.50
20	3	8.90	12.0
Checking system	1	18.80	17.5
	2	23.20	23.0
	3	30.30	28.8

3.2.4 Interferences study

The purpose of this study is to evaluate the interferences of the method. The interference was investigated by adding difference concentration of the interfering substances ca. 5, 10, 20, 50 ppm into 50 ppm ascorbic acid standard solution. The ratio between interference and standard in this study was up to only 1:1 because in the samples, they contain small amount of these substances. The prepared solutions were injected into the system and ratio of the obtained signals compared to the signal of standard solution was used to calculate the recovery percentage. In this study, interfering substances were classified into four different groups including simple sugar, simple organic compound, common ion and heavy metal. The obtained results

are shown in Table 3.6, simple sugar widely found in food plant, e.g., glucose, fructose and sucrose did not appreciably change the recovery of the system similar to citric acid and tartaic acid. Percentages recoveries are 91.5-106.8%. Common cations and anions, e.g. Na^{+}, Ca^{2+}, F^{-} and Cl^{-} did not interfere the purposed method. Percentages recoveries are 98.4-108%. Heavy metal cations like Fe(III), Ce(V), Cu(II) and Fe(II) can oxidize ascorbic acid, therefore they interfere the system. Percentages recoveries are 3.7 -102 %.

Table 3.6 Summary of the percentage of recovery at various ratio of interference to ascorbic acid

Type	Ratio AA : Interference (ppm)	Signal(V)	% Recovery
Sucrose	1 : 0.00	1.084	-
	1 : 0.20	1.058	97.6
	1 : 0.25	1.047	96.5
	1 : 0.50	1.063	98.0
	1 : 1	1.058	97.6
Fructose	1 : 0.00	1.051	-
	1 : 0.20	1.045	99.4
	1 : 0.25	0.9619	91.5
	1 : 0.50	0.9980	94.9
	1 : 1	0.9965	94.8
Glucose	1 : 0.00	1.157	-
	1 : 0.20	1.206	104.2
	1 : 0.25	1.220	105.4
	1 : 0.50	1.203	103.9
	1 : 1	1.148	99.2

Table 3.6 Continued

Type	Ratio AA : Interference (ppm)	Signal(V)	% Recovery
Tartaric acid	1 : 0.00	1.413	-
	1 : 0.20	1.402	99.2
	1 : 0.25	1.435	101.5
	1 : 0.50	1.436	101.7
	1 : 1	1.453	102.9
Citric acid	1 : 0.00	1.385	-
	1 : 0.20	1.479	106.8
	1 : 0.25	1.380	99.7
	1 : 0.50	1.490	107.5
	1 : 1	1.491	107.7
Na^+	1 : 0.00	1.373	-
	1 : 0.20	1.372	99.9
	1 : 0.25	1.398	101.8
	1 : 0.50	1.377	100.3
	1 : 1	1.383	100.8
Ca^{2+}	1 : 0.00	1.406	-
	1 : 0.20	1.402	99.7
	1 : 0.25	1.399	99.5
	1 : 0.50	1.396	99.3
	1 : 1	1.392	99.0
F^-	1 : 0.00	1.392	-
	1 : 0.20	1.392	100
	1 : 0.25	1.376	98.9
	1 : 0.50	1.372	98.5
	1 : 1	1.355	97.3
Cl^-	1 : 0.00	1.374	-
	1 : 0.20	1.352	98.4
	1 : 0.25	1.368	99.6
	1 : 0.50	1.360	99.0
	1 : 1	1.365	99.3

Table 3.6 Continued

Type	Ratio AA : Interference (ppm)	Signal(V)	% Recovery
Ce(IV)	1 : 0.00	1.387	-
	1 : 0.20	1.402	101.1
	1 : 0.25	1.260	90.9
	1 : 0.50	1.209	87.2
	1 : 1	0.726	52.3
Cu(II)	1 : 0.00	1.380	-
	1 : 0.20	0.0511	3.7
	1 : 0.25	-	-
	1 : 0.50	-	-
	1 : 1	-	-
Fe(III)	1 : 0.00	1.395	-
	1 : 0.20	1.428	102.4
	1 : 0.25	1.347	96.6
	1 : 0.50	1.274	91.4
	1 : 1	1.038	74.4
Fe(II)	1 : 0.00	1.367	-
	1 : 0.20	1.363	99.7
	1 : 0.25	1.278	93.4
	1 : 0.50	1.303	95.3
	1 : 1	1.136	83.1

3.3 Sample analysis and comparison to other methods

3.3.1 Comparision with FI-ferrous tartrate method

Following the sample preparation procedure for tea infusion samples in section 2.9.1, twenty tea samples from a local convenient store and local supermarket were analyzed using the proposed method because these tea samples were popular in Thai consumers and the preparation of these samples is very simple. 1.00 g of tea samples were used in sample preparation, if used more than 1.00 g, the content of antioxidant released to the solution was very high. Therefore dilution of the sample is needed which lead to the error in the analysis. And if less than 1.00 g of sample was used, the

content of antioxidant is too small which may not be representative of all samples. Antioxidant contents (ascorbic acid equivalent) were calculated by using calibration graph equation that was plotting between peak height obtained and concentration of ascorbic acid. The same samples were also analyzed by FI-ferrous tartrate method as described in section 2.9.3 for comparison. The obtained results are shown in table 3.7. In the results, they gave a difference value because in tea samples there are a variety of antioxidants that they can react more or less with triiodide ion than ferrous tartrate. So the values are different for comparison of the results they should be compared the trend more than the obtained value. The correlation plot of results from the proposed method versus FI-ferrous tartrate method is shown in Figure 3.7. It was found that the results from the proposed method and FI-ferrous tartrate method were in good correlation which considered the correlation coefficient: r^2 if it gave correlation closed to 1.000, showed that the two methods have a corresponding result. FI-ferrous tartrate method and the proposed method gave $r^2 = 0.961$.

Table 3.7 Antioxidant content in some tea infusion samples.

Sample	Description of sample	FI-amp (ascorbic acid eq)	FI-ferrous tartrate method (tannin eq)
1	Mulberry (Herbal Horse 2 Thailand)	3.8 ± 0.1	23.3 ± 0.1
2	Chinese Tea No.1 (Big C Thailand)	12.9 ± 0.1	83.0 ± 0.1
3	Jasmine Tea (Three Horses Tea Thailand)	17.5 ± 0.2	123.5 ± 0.1
4	Chinese Tea No. 1 (Three Horses Tea Thailand)	15.9 ± 0.2	99.3 ± 0.1
5	Chinese Tea No. 3 (Three Horses Tea Thailand)	14.0 ± 0.2	82.1 ± 0.1

Table 3.7 Continued

Sample	Description of sample	FI-amp (ascorbic acid eq)	FI-ferrous tartrate method (tannin eq)
6	Oolong Tea (Sum Siew Tea leaf Thailand)	14.2 ± 0.1	79.7 ± 0.1
7	Sumsiew Hom Tea No.1 (Sum Siew Tea leaf Thailand)	14.7 ± 0.3	75.1 ± 0.1
8	Jasmine Tea No.1 (Sum Siew Tea leaf Thailand)	16.7 ± 0.2	111.2 ± 0.1
9	Green Calabash Tea leaf (Sum Siew Tea leaf Thailand)	13.7 ± 0.2	84.4 ± 0.1
10	Poy Sien Tea leaf (Sum Siew Tea leaf Thailand)	5.9 ± 0.1	28.3 ± 0.1
11	Mulberry green tea, (Renongtea)	0.56 ± 0.1	11.1 ± 0.1
12	Ginkgo Leaves Tea (Dr.Green)	0.8 ± 0.1	5.9 ± 0.1
13	Thai traditional tea herb	12.5 ± 0.1	65.7 ± 0.1
14	Roselle Tea (Chao Phya Abhalbhubejhr)	0.7 ± 0.1	23.4 ± 0.1
15	Safflower – Garcinia Tea (Thanyaporn heab)	1.2 ± 0.1	10.5 ± 0.1
16	Jiaogulan (Mei-Fong Tea, Chiang Rai)	1.4 ± 0.2	4.9 ± 0.1
17	Moringa Tea with peppermint (Pumedin natural product, Chiang Mai	0.7 ± 0.1	4.5 ± 0.1
18	Safflower tea (Herb Basic, Chiang Mai)	1.2 ± 0.4	10.2 ± 0.1
19	Jiaogulan (Herb Basic, Chiang Mai)	1.0 ± 0.1	6.9 ± 0.1
20	Mulberry green tea (Herb Basic, Chiang Mai)	0.4 ± 0.1	9.0 ± 0.1

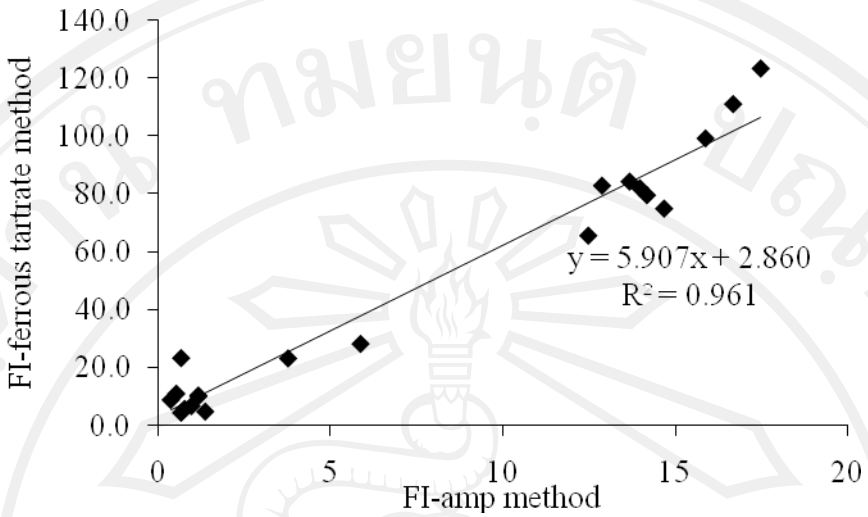

Figure 3.7 Correlation graph of antioxidant contents obtained by the proposed method and FI-ferrous tartrate method

3.4.2 Comparision with FI – colorimetric method based on FRAP reaction

Following the sample preparation procedure for tea infusion samples in section 2.9.1, twenty tea samples from a local convenient store and local supermarket were analyzed using the proposed method because these tea samples were popular in Thai consumers and the preparation of these samples very simple. 1.00 g of tea samples were used in sample preparation, if used more than 1.00 g, the content of antioxidant was very high. They used to dilute the sample for analysis may be causing the error. And if used less than 1.00 g, the content of antioxidant is too small which may not be representative of all samples. Antioxidant contents (ascorbic acid equivalent) were calculated by using calibration graph equation that was plotting between peak height obtained and concentration of ascorbic acid. The same samples were also analyzed by FI – colorimetric method based on FRAP reaction as describe in section 2.9.4 for

comparison. The obtained results are shown in table 3.8. In the results, they gave a difference value because in tea samples there are a variety of antioxidants that they can react more or less with triiodide ion than Fe(III). So the values are different for comparison of the results they should be compare the trend more than the obtained value. The correlation plot of the proposed method and FI – colorimetric method based on FRAP reaction is shown in Figure 3.8. It was found that the results from the proposed method and FI – colorimetric method based on FRAP reaction were in good correlation which considered the correlation coefficient: r^2 if it gave correlation closed to 1.000 showed that the two methods have a corresponding result. FI – colorimetric method based on FRAP reaction and the proposed method which gave $r^2 = 0.919$.

Table 3.8 Antioxidant content in some tea infusion samples.

Sample	Description of sample	FI-Amp (ascorbic acid eq)	FI – colorimetric method based on FRAP reaction (ascorbic acid eq)
1	Mulberry (Herbal Horses 2 Thailand)	3.8 ± 0.1	30.2 ± 0.1
2	Chinese Tea No.1 (Big C Thailand)	12.9 ± 0.1	57.4 ± 0.1
3	Jasmine Tea (Three Horses Tea Thailand)	17.5 ± 0.2	61.6 ± 0.1
4	Chinese Tea No. 1 (Three Horses Tea Thailand)	15.9 ± 0.2	51.1 ± 0.1
5	Chinese Tea No. 3 (Three Horses Tea Thailand)	14.0 ± 0.2	63.2 ± 0.1
6	Oolong Tea (Sum Siew Tea leaf Thailand)	14.2 ± 0.1	64.3 ± 0.1
7	Sumsiew Hom Tea No.1 (Sum Siew Tea leaf Thailand)	14.7 ± 0.3	55.0 ± 0.1

Table 3.8 Continued

Sample	Description of sample	FI-amp (ascorbic acid eq)	FI – colorimetric method based on FRAP reaction (ascorbic acid eq)
8	Jasmine Tea No.1 (Sum Siew Tea leaf Thailand)	16.7 ± 0.2	53.1 ± 0.1
9	Green Calabash Tea leaf (Sum Siew Tea leaf Thailand)	13.7 ± 0.2	52.4 ± 0.1
10	Poy Sien Tea leaf (Sum Siew Tea leaf Thailand)	5.9 ± 0.1	36.2 ± 0.1
11	Mulberry green tea, (Renongtea)	0.56 ± 0.1	1.9 ± 0.1
12	Ginkgo Leaves Tea (Dr.Green)	0.8 ± 0.1	7.0 ± 0.1
13	Thai traditional tea herb	12.5 ± 0.1	42.2 ± 0.1
14	Roselle Tea (Chao Phya Abhalbhubejhr)	0.7 ± 0.1	10.7 ± 0.1
15	Safflower – Garcinia Tea (Thanyaporn heab)	1.2 ± 0.1	4.1 ± 0.1
16	Jiaogulan (Mei-Fong Tea, Chiang Rai)	1.4 ± 0.2	0.6 ± 0.1
17	Moringa Tea with peppermint (Pumedin natural product, Chiang Mai	0.7 ± 0.1	1.2 ± 0.1
18	Safflower tea (Herb Basic, Chiang Mai)	1.2 ± 0.4	3.5 ± 0.1
19	Jiaogulan (Herb Basic, Chiang Mai)	1.0 ± 0.1	3.1 ± 0.1
20	Mulberry green tea (Herb Basic, Chiang Mai)	0.4 ± 0.1	8.9 ± 0.1

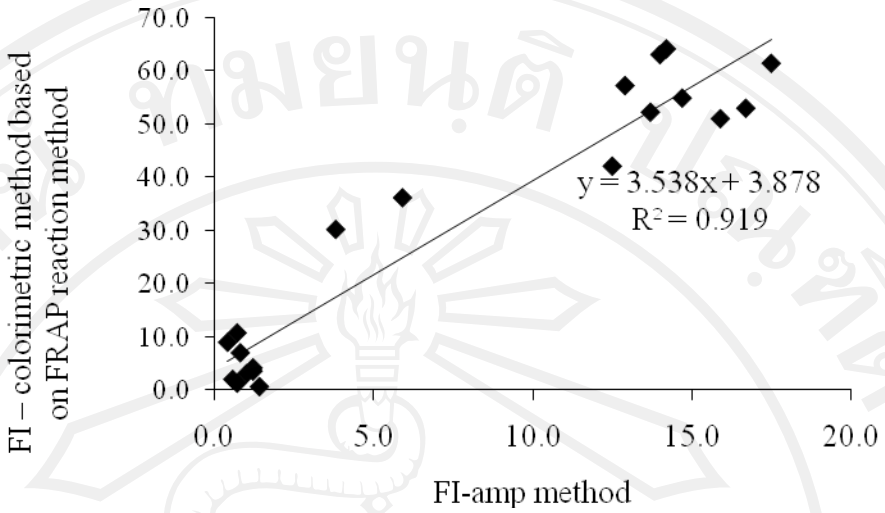

Figure 3.8 Correlation graph of antioxidant contents by the proposed method and FI – colorimetric method based on FRAP reaction method.