CHAPTER 2

EXPERIMENT

2.1 Chemicals

All chemicals used are analytical reagent grade and all standard and reagent solutions were prepared with ultrapure water (Milli Q water, resistivity of 18.2 MΩ cm^{-1}) obtained from a Millipore water purification system (Millipore, Sweden). Chemicals used in this research are listed below:

1. Ascorbic acid : $C_6H_8O_6$, (Merck, Germany)
2. Potassium iodate : KIO_3 (Fluka, Switzerland)
3. Potassium iodide : KI (BHD, England)
4. Hydrochloric acid : HCl (Lab Scan, Thailand)
5. Ferrous sulphate : $FeSO_4.7H_2O$ (Carlo Erba, Italy)
6. Ceric ammonium nitrate $(NH_4)_2Ce(NO_3)_6$ (Ajax Finechem, Australia)
7. Iron (III) chloride hexahydrate : $FeCl_3.6H_2O$ (POCH SA, Poland)
8. Sodium chloride : NaCl (TRS, USA)
9. Calcium fluoride : CaF_2 (Riedel-de Haen, USA)
10. Citric acid monohydrate : $C_6H_8O_7.H_2O$ (Merck, Germany)
11. L(+) - Tartaric acid : $C_4H_6O_6$ (Carlo Erba, Italy)
12. D(+) – Fructose : $C_6H_{12}O_6$ (Fluka, Switzerland)
13. D(+) – Glucose : $C_6H_{12}O_6$ (Fisher, USA)
14. Sucrose : $C_{12}H_{22}O_{11}$ (Ajax Finechem, Australia)

2.2 Material and instruments

1. Peristaltic pump (Ismatec, Switzerland)
2. Tygon pump tubing (Ismatec, Switzerland)
3. PTFE tubing (1.59 mm OD, 0.51 mm ID) (Upchurch Scientific, USA)
4. Six – port injection valve (Upchurch Scientific, USA)
5. Flow through electrochemical cell (Cross-flow cell, Model MF – 1093, BAS, USA) composes of a 3 mm diameter glassy carbon working electrode (GCE), a Ag/AgCl reference electrode (RE) and a stainless steel auxiliary electrode (AE)
6. Homemade amperometric detector
7. Digital multimeter (UNI-T UT33D, Hong Kong)
8. Analytical balance (Mettler Toledo, Switzerland)
9. Electrode polishing kit PK-4 MF-2060

2.3 Software

1. Microsoft Excel 2007 (Microsoft, USA)
2. eDAQ Chart (eDAQ, Australia)

2.4 Preparation of Standard solutions and reagents

2.4.1 Working standard solution of ascorbic acid

Working standard solutions (8-100 ppm) was prepared by appropriate dilution of the stock ascorbic acid solution with DI water. Stock ascorbic acid solution (1000 ppm) was prepared by dissolving 0.1000 g of ascorbic acid in 100 mL of DI water.

2.4.2 Working potassium iodate solution 25 ppm in hydrochloric acid 0.2 M

Working potassium iodate solution 25 ppm in hydrochloric acid 0.2 M was prepared by appropriate dilution of the stock potassium iodate solution and conc. hydrochloric acid l with DI water. Stock potassium iodate solution (1000 ppm) was prepared by dissolving 0.1251 g of potassium iodate in 100 mL of DI water

2.4.3 Working potassium iodide solution 0.005 M

Working potassium iodide solution 0.005 M was prepared by appropriate dilution of the stock potassium iodide solution with DI water. Stock potassium iodide solution (0.1 M) was prepared by dissolving 1.6668 g of potassium iodide in 100 mL of DI water.

2.5 Preparation of electrode

The glassy carbon electrode was polished with water slurry of 1 μm alumina polish (Al_2O_3) on a polishing pad and polished about 2 min to obtain a fresh surface.

2.6 Manifold of flow injection amperometric method using triiodide as a reagent for estimation of antioxidant activity

Flow injection amperometric method using triiodide as a reagent for estimation of antioxidant activity is shown in Figure 2.1. The system is comprised of peristaltic pump (Ismatec, Switzerland) with pump tubing, six port injection valve (Upchurch Scientific, USA), a mixing coil (PTFE, i.d. 0.5 mm), homemade amperometric detector with Flow through electrochemical cell (Cross-flow cell, Model MF – 1093, BAS, USA) composes of a 3 mm diameter glassy carbon working electrode (GCE), a

Ag/AgCl reference electrode (RE) and a stainless steel auxiliary electrode (AE) and the system was controlled by a personal computer using a software program written in-house.

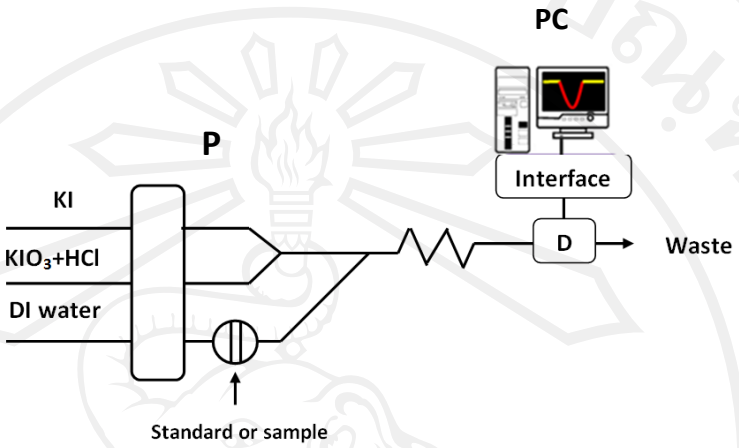

Figure 2.1 Manifold for the estimation of antioxidant activity by the proposed method. P = Pump, D = amperometric detector, PC = Computer.

Figure 2.1 shows a manifold for the estimation of antioxidant activity. Iodate reacts with iodide in acidic medium producing triiodide which undergo electrochemical reduction on a glassy carbon working electrode at 200 mV versus Ag/AgCl reference electrode producing baseline electrical current. When standard or sample solution was injected into the system, antioxidant reacts with triiodide leading to the decrease in the electrical current which was directly proportional to antioxidative activity which was recorded as FIA peak.

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

The optimization of flow injection amperometric method using triiodide as reagent was performed by univariate method, one parameter was varied while the

others were kept constant. The series of ascorbic standard solutions was injected into the system and considering for the highest value of a slope of a calibration graph. The slopes of the calibration graphs on the study of some parameters are described below.

2.7.1 Concentration of potassium iodate

The studied concentrations of iodate are 15, 25, 35, 50 and 75 ppm. The optimal concentration was chosen by considering slope of calibration graphs of standard ascorbic acid in the range of 8-100 ppm. The conditions for the study are summarized in Table 2.1.

Table 2.1 The conditions for the study of effect of potassium iodate concentation

Parameter	**Value**
Potassium Iodide	1 mM
Hydrochloride acid	0.1 M
Total flow rate	3.2 $ml.min^{-1}$
Sample volume	100 µL
Mixing coil length	50 cm
Electrode	GCE
Electrode potential	200 mV versus Ag/AgCl

2.7.2 Concentration of potassium iodide

Effect of potassium iodide concentration was investigated by varying concentration of potassium iodide at 0.1, 0.5, 1, 5 and 10 mM. The optimal concentration was chosen by considering slope of calibration graphs of standard ascorbic acid in the range of 8-100 ppm. The conditions for the study are summarized in Table 2.2.

Table 2.2 The conditions for the study of effect of potassium iodide concentation

Parameter	**Value**
Potassium Iodate	25 ppm
Hydrochloride acid	0.1 M
Total flow rate	3.2 $ml.min^{-1}$
Sample volume	100 µL
Mixing coil length	50 cm
Electrode	GCE
Electrode potential	200 mV versus Ag/AgCl

2.7.3 Concentration of hydrochloric acid

The studied concentration range of potassium iodide was 0.010, 0.020, 0.050, 0.100, 0.200, 0.500 and 1.000 M. The optimal concentration was chosen by considering slope of calibration graphs of standard ascorbic acid in the range of 8-100 ppm. The conditions for the study are summarized in Table 2.3.

Table 2.3 The conditions for the study of effect of hydrochloric acid concentration

Parameter	**Value**
Potassium iodate	25 ppm
Potassium iodide	5 mM
Total flow rate	3.2 $ml.min^{-1}$
Sample volume	100 µL
Mixing coil length	50 cm
Electrode	GCE
Electrode potential	200 mV versus Ag/AgCl

2.7.4 Total flow rate

The influence of the total flow rate was investigated at 2.0, 2.7, 3.3, 4.0 and 4.5 $ml.min^{-1}$. The optimal concentration was chosen by considering slope of calibration graphs of standard ascorbic acid in the range of 8-100 ppm. The conditions for the study are summarized in Table 2.4.

Table 2.4 The conditions for the study of effect of total flow rate

Parameter	Value
Potassium iodate	25 ppm
Potassium iodide	5 mM
Hydrochloric acid	0.2 M
Sample volume	100 µL
Mixing coil length	50 cm
Electrode	GCE
Electrode potential	200 mV versus Ag/AgCl

2.8 Analytical characteristics of the procedure

2.8.1 Calibration curves and limit of detection

The optimum conditions of FI-amperometric method were used to construct the calibration graph by injection a series of standard ascorbic acid (8-100 ppm) into the system. The calibration graph was plotting between peak height and concentration of ascorbic acid. The limit of detection is calculated from $\frac{3S_a}{b}$ 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].

2.8.2 Precision study

The precision of the method was investigated by nine – replicated injection of ascorbic acid at low and high concentration level ca. 8 and 100 ppm. The percentage of relative standard deviation (%RSD) values was used for evaluating the precision and can be calculated from the equation (2.1).

$$\%RSD = \frac{SD \times 100}{\bar{x}} \quad (2.1)$$

When: $\%RSD$ = percentage relative standard deviation

SD = standard deviation

$\bar{x}$ = mean

2.8.3 Recovery study

The 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 from calculation. The concentration of ascorbic acid before and after spiking, and expected value were used to calculate percentage recovery.

2.8.4 Interferences study

Interference study was performed by adding different concentrations of the interference ca. 5, 10, 20, 50 ppm into 50 ppm ascorbic acid standard solution. 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.

2.9 Sample analysis

2.9.1 Preparation of sample

1.00 g of tea samples were boiled in 50 ml water for 30 min. Then, the solution was filtered through Whatman filter paper No. 1 and adjusted the volume of sample to 50 ml [1].

2.9.2 Precision study of sample extraction method

The precision of the sample extraction method was investigated by fifteen – replicated injection of three samples at low, medium and high content of antioxidant. The percentage of relative standard deviation (%RSD) values was used for evaluating the precision of the sample extraction method.

2.9.3 Comparison with FI – ferrous tartrate method

The antioxidant (tannin equivalent) react with ferrous tartarte (solution 0.5 mL of ferrous tartrate solution comprising 0.1 g of ammonium ferrous sulfate hexahydrate plus 0.5 g of potassium sodium (+)-tartrate tetrahydrate dissolved in 100 mL of

deionized water) and 2.5 mL phosphate buffer (0.1 M pH 8) to produce a colored complex that measured the absorbance at 540 nm.

2.9.4 Comparison with FI – colorimetric method based on FRAP reaction method

The antioxidant (ascorbic acid equivalent) react with Fe(III)- 1,10-phenanthroline complex to Fe(II) - 1,10-phenanthroline. Reagent of Fe(III)-1,10-phenanthroline solution was prepared by dissolving 0.0720 g of ammonium iron(III) sulfate in DI water and added 1.0 M hydrochloric acid 2 ml then mixed the solution with 1,10-phenanthroline solution and adjusting to the volume of 100 mL. The colorimetric detective of Fe(II) – 1,10-phenanthroline complex at 540 nm.

2.9.5 Application of the method to real samples

The flow injection amperometric system was used to estimate antioxidant (ascorbic acid equivalent) in tea infusion samples. The FI-amperometric method as described above, a standard / sample solution (100 μL) was injected into a water carrier stream and mixed in-line with the reagent solution. The antioxidants react with triiodide which was produced by the reaction of iodate with iodide in acidic medium leading to the decrease in the amperometric current that was directly proportion to antioxidative activity. The output signal from the detector was recorded as FIA peak on a personal computer. Peak height was directly proportional to concentration of ascorbic acid. A calibration graph was constructed by plotting versus ascorbic acid concentration and it was used for quantification of the antioxidant content in sample. The results obtained from the proposed were compared with those of FI-ferrous

tartrate method and FI- colorimetric method based on FRAP reaction which are the reference methods for estimation of ascorbic acid. The correlation plots were employed to examine the correlation between the proposed and reference methods.