CHAPTER 3

RESULTS AND DISCUSSION

3.1 Asparagus based amperometric sensor for fluoride determination

In this section, an asparagus based amperometric sensor has been described for fluoride determination using a static method. Optimum conditions for fluoride determination were achieved. The proposed method was applied successfully for the determination of fluoride in some commercial fluoride tablets collected in the chemist shop in Amphur Muang, Changwat Chiangmai. Satisfactory results were obtained. Evaluation of the proposed method was also carried out by comparing the results obtained with those obtained by potentiometric measurement with fluoride selective electrode [65]. It was found that results obtained by both methods were in good agreement using t-test.

3.1.1 Cyclic voltammetry

Initially, cyclic voltammograms (Figure 3.1) for H_2O_2 bioelectrode in 0.05 M NaH_2PO_4-NaOH buffer pH 7.0 for; A, plain carbon paste electrode; B, ferrocene (5% w/w)-modified electrode; C, asparagus tissue (6% w/w) and ferrocene-modified (5% w/w) carbon paste bioelectrode; scan rate, 0.03 V s^{-1}; D, in the presence of 2.0 mM H_2O_2 were recorded in order to evaluate the performance of the propose method.

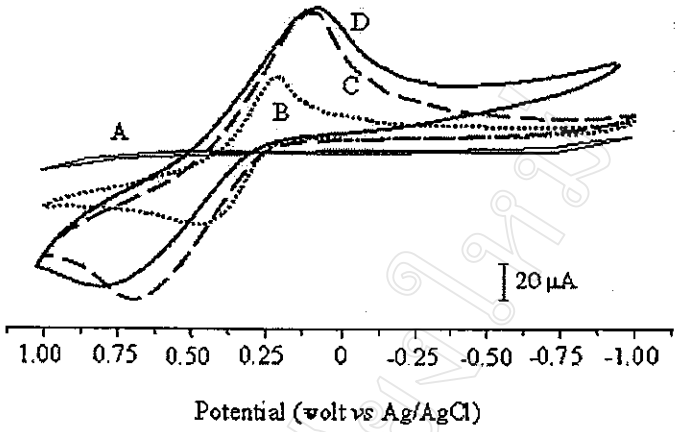

Figure 3.1 Cyclic voltammogram for H_2O_2 bioelectrode

[0.05 M NaH_2PO_4-NaOH buffer pH 7.0 for; (A) plain carbon paste electrode; (B) ferrocene (5% w/w)-modified electrode; (C) asparagus tissue (6% w/w) and ferrocene-modified (5% w/w) carbon paste bioelectrode, scan rate, 0.03 V s^{-1}; (D) in the presence of 2 mM H_2O_2]

Figure 3.1 shows cyclic voltammogram obtained with a plain carbon paste electrode (A) and a ferrocence-modified carbon paste electrode (B). It can be seen that no redox wave was observed for a plain carbon paste electrode, whereas well-defined cathodic and anodic waves appeared at ferrocene-modified carbon paste electrode. Accordingly, the wave appearing at the latter electrode was ascribed to the electrochemical redox reaction of ferrocene molecules that had been mixed in the carbon paste electrode. The addition of ground asparagus (*Asparagus officinalis*) skin to the ferrocene-modified paste showed little effect on the redox peak potential except that a larger current was observed, indicating an enhanced diffusional flux (C). The larger current obtained for the tissue-modified carbon paste compared with those of the ferrocene-modified carbon paste indicated that the former electrode is more hydrophobic than the latter electrode, thus, allowing a large surface exposure. The voltammetry of the proposed bioelectrode in the presence of H_2O_2 (D) clearly exhibits an increase in the reduction current of the ferricinium

ion and a decrease in the oxidation current of ferrocene. The bioelectrode mechanism can be summarized as shown in Figure 3.2. The existing peroxidase (reduced form) in asparagus tissue [30] reduces H_2O_2 to H_2O, forming the oxidized species, which then oxidizes the ferrocene used as a mediator to the ferricinium ion. The ferricinium ion is subsequently reduced back to ferrocene by rapid reaction involving the acceptance of an electron from the electrode.

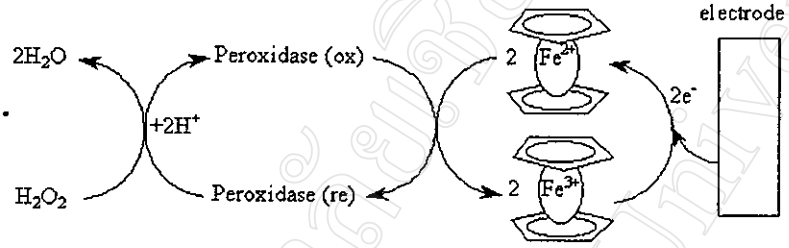

Figure 3.2 Reaction sequence within the asparagus tissue-based and ferrocene-mediated bioelectrodes [30]

3.1.2 Optimization of experimental variables in batch system

3.1.2.1 Effect of pH in batch system

The pH dependence of the bioelectrode over the pH range 4.0 to 8.0 is illustrated in Table 3.1 and Figure 3.3. The resulting peak-shaped pH profile showed maximum sensitivity of the bioelectrode response at pH 4.5. However, it was found that the noise level gradually increased with decreasing pH. An optimum pH range of 3.8 to 4.8 has been recently reported for asparagus peroxidase [72]. This indicates that the pH profile is governed by the enzymatic activity. Based on the results obtained, a phosphate buffer solution of pH 5.0 was selected for subsequent studies as a compromise between bioelectrode sensitivity and signal to noise characteristic [30].

Table 3.1 Effect of pH on the sensitivity of the bioelectrode in batch system

pH	Current (μA)* at $[H_2O_2]$ (mM)			Sensitivity** ($\mu A\ mM^{-1}$)
	0.1	0.2	0.3	
4.0	0.123	0.202	0.264	0.943
4.5	0.130	0.223	0.313	1.083
5.0	0.137	0.233	0.303	1.081
5.5	0.140	0.223	0.290	1.041
6.0	0.113	0.180	0.237	0.845
6.5	0.090	0.153	0.197	0.705
7.0	0.067	0.117	0.157	0.550
7.5	0.063	0.113	0.157	0.543
8.0	0.077	0.123	0.160	0.574

* = Mean of triplicate

** = The sensitivity is defined as the slope ($\Delta I/[H_2O_2]$) of a plot of response *versus* hydrogen peroxide concentration

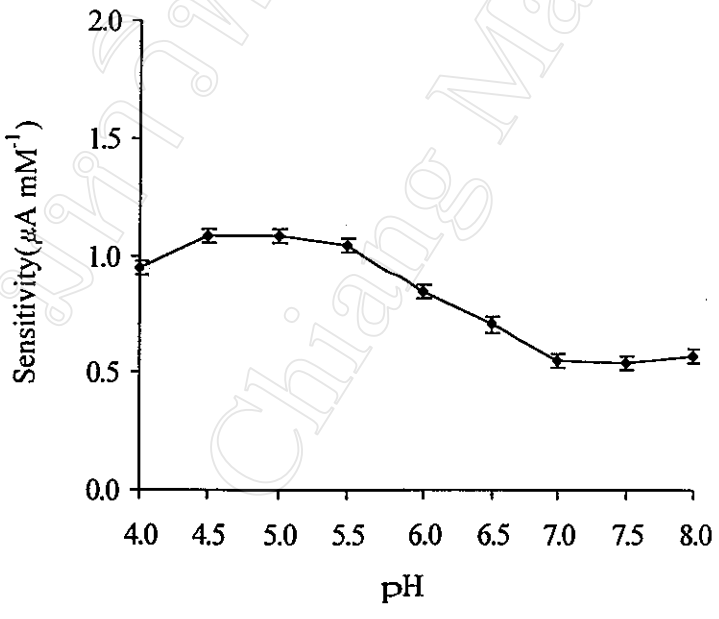

Figure 3.3 Effect of pH on the sensitivity of the bioelectrode in batch system

[5% (w/w) asparagus tissue and 4% (w/w) ferrocene; 10.0 ml of 0.05 M NaH_2PO_4-NaOH buffer; applied potential, 0.00 V (*vs.* Ag/AgCl)]

3.1.2.2 Effect of applied potential in batch system

The effect of applied potential on the bioelectrode sensitivity is shown in Table 3.2 and Figure 3.4. It was found that the bioelectrode sensitivity decreased slightly as the potential was changed from –0.20 to +0.10 V *versus* Ag/AgCl. As the potential approached 0.15 V, the noise increased drastically. This behavior is ascribed to the electrochemical oxidation of the ferrocene contained in the paste occurring in this potential range. At –0.20 V, the cathodic current kept increasing even without injecting H_2O_2. This behavior was also reported by previous workers [18] and was ascribed to the reduction of dissolved oxygen at this potential. A potential of –0.05 V was selected for the remainder of the experiments as the best compromise between the resulting signal and the noise level

Table 3.2 Effect of applied potential on the sensitivity of the bioelectrode in batch system

Applied potential (V)	**Current (μA)* at $[H_2O_2]$ (mM)**			**Sensitivity** (μA mM^{-1})**
	0.1	**0.2**	**0.3**	
-0.20	0.237	0.397	0.530	1.81
-0.15	0.183	0.307	0.390	1.40
-0.10	0.160	0.263	0.347	1.23
-0.50	0.140	0.240	0.317	1.12
0.00	0.130	0.220	0.293	1.04
+0.05	0.127	0.213	0.277	0.99
+0.10	0.123	0.210	0.271	0.97

* = Mean of triplicate

** = The sensitivity is defined as the slope ($\Delta I/[H_2O_2]$) of a plot of response *versus* hydrogen peroxide concentration

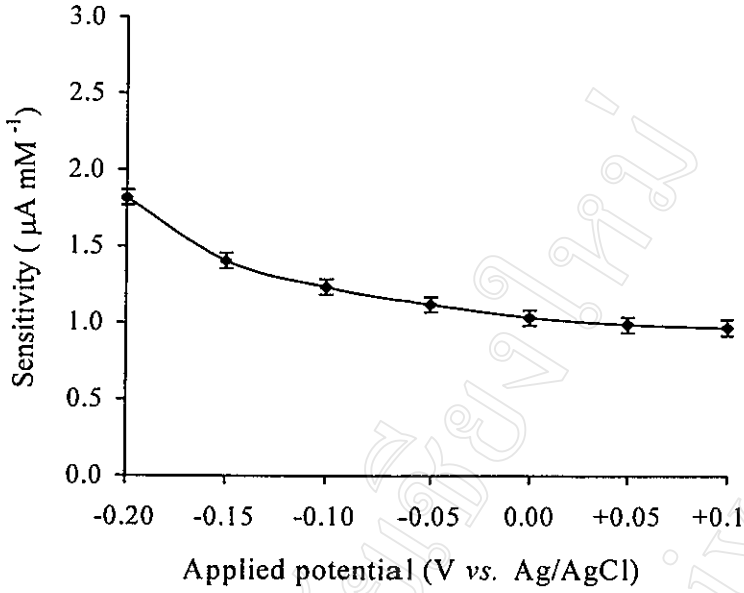

Figure 3.4 Effect of applied potential on the sensitivity of the bioelectrode in batch system [5%w/w asparagus tissue and 4%w/w ferrocene; 10.0 ml of 0.05 M NaH_2PO_4-NaOH buffer (pH 5.0)]

3.1.2.3 Effect of tissue loading in batch system

The tissue loading has a profound effect upon the bioelectrode response to H_2O_2. Increasing the tissue composition of the bioelectrode from 3 to 8%(w/w) resulted in an increase in the bioelectrode sensitivity (Table 3.3 and Figure 3.5), reflecting the increase in the biocatalytic activity. However, the response times as well as the noise level gradually increased with the increase in tissue content. The sensitivity of the bioelectrode decreased when the tissue loading was more than 9%(w/w). This behavior is ascribed to the lowering of the electrical conductivity due to the reduction in graphite loading. A tissue loading of 7%(w/w) was selected as the best compromise between the resulting signal and the noise level [30].

Table 3.3 Effect of the amount of asparagus tissue on the sensitivity of the bioelectrode in batch system

Tissue loading (%w/w)	Current (μA)* at $[H_2O_2]$ (mM)			Sensitivity** (μA mM^{-1})
	0.1	0.2	0.3	
3	0.123	0.220	0.330	1.11
5	0.240	0.485	0.705	2.37
6	0.410	0.700	1.050	3.54
7	0.413	0.809	1.120	3.85
8	0.410	0.790	1.160	3.90
9	0.410	0.775	1.120	3.80
10	0.370	0.725	1.070	3.59
11	0.360	0.702	0.986	3.37
13	0.250	0.590	0.835	2.81

* = Mean of triplicate

** = The sensitivity is defined as the slope ($\Delta I/[H_2O_2]$) of a plot of response *versus* hydrogen peroxide concentration

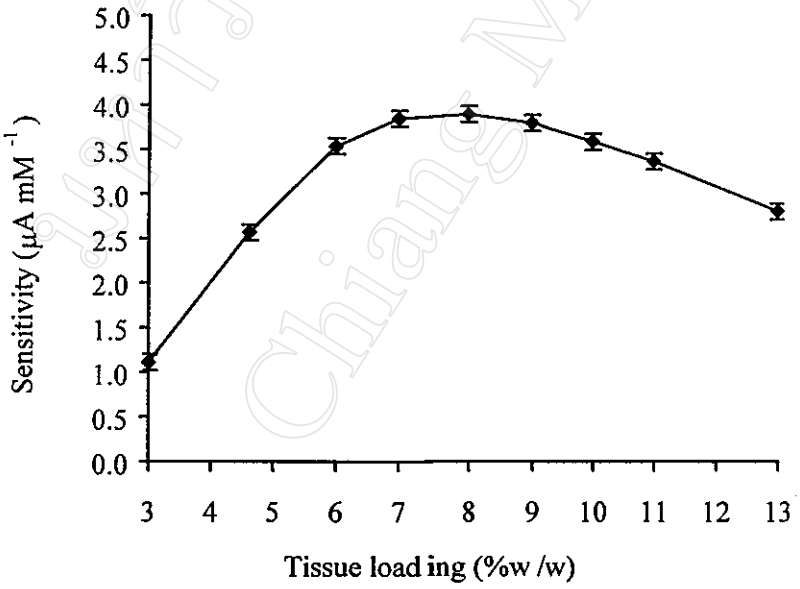

Figure 3.5 Effect of amount of asparagus tissue on the sensitivity of the bioelectrode in batch system [4% (w/w) ferrocene; 10.0 ml of 0.05NaH_2PO_4-NaOH buffer (pH 5.0); applied potential, -0.05 V (*vs.* Ag/AgCl)]

3.1.2.4 Effect of mediator loading in batch system

Table 3.4 and Figure 3.6. illustrates signals increasing substantially with increase in mediator loading from 2 to 6%(w/w). Further increase in mediator loading above 6%(w/w) resulted in a decrease in the bioelectrode responses. Such a decrease when a larger amount of mediator is used is a typical behavior of a mediator-based sensor and is ascribed to the lowering of the electrical conductivity due to the reduction in graphite loading [30]. Moreover, the noise increased dramatically with increase in mediator loading above 6%(w/w). Thus, a mediator loading of 6%(w/w) was selected for further experiments.

Table 3.4 Effect of ferrocene loading on the sensitivity of the bioelectrode in batch system

Ferrocene loading (%w/w)	**Current (μA)* at $[H_2O_2]$ (mM)**			**Sensitivity** (μA mM^{-1})**
	0.1	**0.2**	**0.3**	
0	0.060	0.125	0.180	0.61
2	0.330	0.635	0.900	3.07
4	0.400	0.820	1.210	4.05
5	0.480	0.870	1.300	4.37
6	0.480	0.920	1.350	4.55
7	0.460	0.860	1.300	4.34
8	0.390	0.813	1.201	4.01
10	0.310	0.645	0.905	3.08

* = Mean of triplicate

** = The sensitivity is defined as the slope ($\Delta I/[H_2O_2]$) of a plot of response *versus* hydrogen peroxide concentration

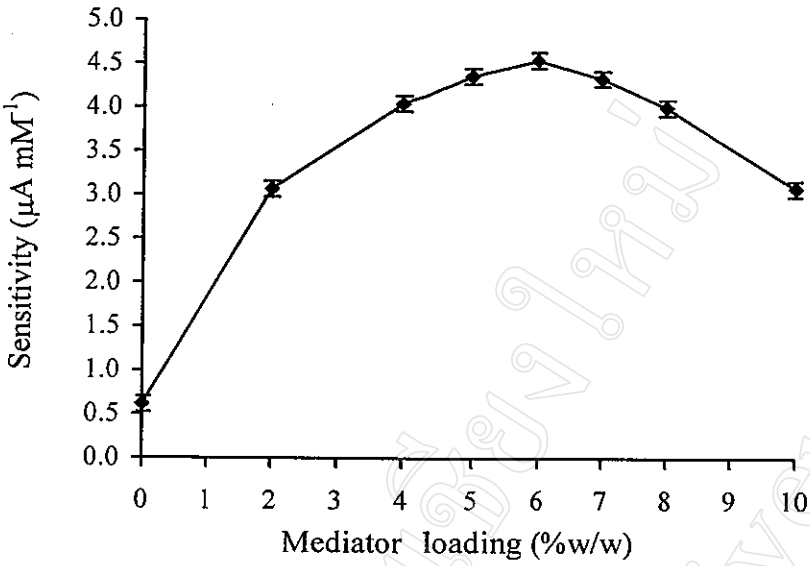

Figure 3.6 Effect of ferrocene loading on the sensitivity of the bioelectrode in batch system [7%(w/w) asparagus tissue; 10.0 ml of 0.05 M NaH_2PO_4-NaOH buffer (pH 5.0); applied potential, -0.05 V (*vs.* Ag/AgCl)]

3.1.2.5 Effect of H_2O_2 concentration in batch system

The effect of H_2O_2 concentration on the fluoride sensors is shown in Table 3.5 and Figure 3.7. It was seen that the responses to fluoride increased with increase in H_2O_2 concentration ranging from 0.05 to 0.1 mM and decreased with increase in H_2O_2 concentration above 0.2 mM. This behavior is in good agreement with the competitive inhibition kinetic [73]. In fact, if the H_2O_2 concentration is low, fluoride will compete favorably with the H_2O_2 for the binding sites on the enzyme and the degree of inhibition will be greater. In contrast, if the H_2O_2 concentration is high, the fluoride will be much less successful in competing with H_2O_2 for the available binding sites and the degree of inhibition will be less marked. A constant of H_2O_2 concentration of 0.1 mM was selected for the remainder of the experiments.

Table 3.5 Effect of H_2O_2 concentration on the bioelectrode in batch system

$[F^-]$ (mg l^{-1})	Current (nA)* at $[H_2O_2]$(mM)					
	0.05	**0.10**	**0.20**	**0.30**	**0.40**	**0.50**
0.0	0.00	0.00	0.00	0.00	0.00	0.00
4.0	28.67	32.00	31.33	22.00	17.67	23.00
8.0	48.00	55.67	53.20	36.33	33.00	36.00
12.0	61.67	73.33	72.00	48.33	46.00	47.67
16.0	74.67	87.67	86.00	59.67	55.00	56.33
20.0	85.67	101.00	101.67	70.00	65.67	62.67

* = Mean of triplicate

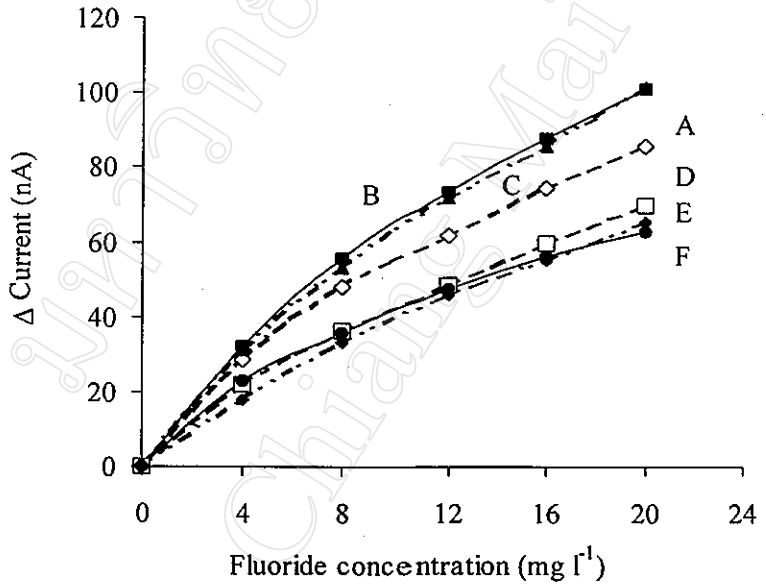

Figure 3.7 Effect of H_2O_2 concentration on the bioelectrode in batch system

[(A) 0.05; (B) 0.1; (C) 0.2; (D) 0.3; (E) 0.4 and (F) 0.5 mM H_2O_2; 7% (w/w) asparagus tissue and 6% (w/w) ferrocene) in a 10.0 ml of 0.05 M NaH_2PO_4-NaOH buffer (pH 5.0); applied potential, -0.05 V (*vs.* Ag/AgCl)]

3.1.2.6 Summary of the optimum conditions in batch system

The experimental variables of asparagus tissue-based amperometric sensors for fluoride determination had been optimized. The variables, their ranges studied and optimum conditions are shown in Table 3.6.

Table 3.6 Experimental variables, their ranges studied and optimum values of fluoride sensor

Experimental variables	Range studied	Value chosen
pH	4.0 - 8.0	5.0
Applied potential (V) (*versus* Ag/AgCl)	-0.20 to +0.10	-0.05
Tissue loading (%w/w)	3 – 11	7
Ferrocene loading (%w/w)	0 – 8	6
H_2O_2 concentration (mM)	0.05 - 0.50	0.1

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3.1.3 Analytical characteristics of bioelectrode in batch system

3.1.3.1 Calibration, detection limit and reproducibility

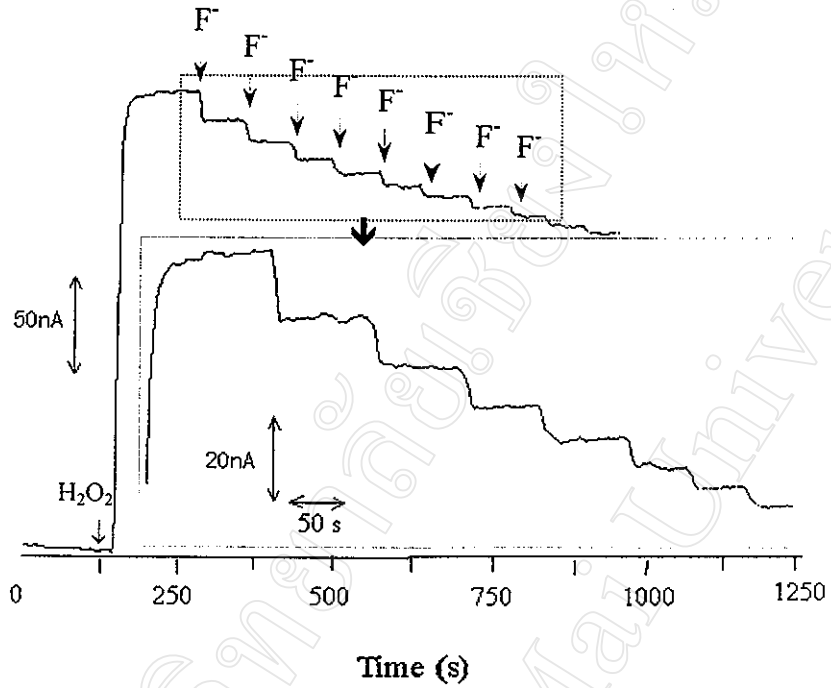

Figure 3.8 Typical current-time recording of fluoride in batch system [stepwise addition, 2 mg l^{-1} of fluoride; 7%(w/w) asparagus tissue and 6%(w/w) ferrocene; 10.0 ml of 0.05 M NaH_2PO_4-NaOH buffer (pH5.0) containing 0.1 mM H_2O_2; applied potential, -0.05 V (*vs.* Ag/AgCl)]

Figure 3.8 shows the typical current *versus* time responses obtained using the bioelectrode under the optimum experimental conditions. A calibration graph for fluoride over the concentration range of 0.0 to 20.0 mg l^{-1} was obtained. It was found that the bioelectrode exhibited an almost linear in calibration up to 14.0 mg l^{-1} (Table 3.7 and Figure 3.9). The linear regression analysis current (y) *versus* F^- concentration (x) yielded the regression equation as follows:

$$y = 8.2\text{x}10^{-3}\ x \qquad (r = 0.997) \qquad (3.1)$$

The sensitivity (slope of the initial linear range) corresponds to 8.2 nA/mg l^{-1} ($8.2x10^{-3}$ μA/mg l^{-1}). The signal-to-noise characteristics (*S/N*)=3 indicates a detection limit of 0.5 mg l^{-1} fluoride. The response is highly reproducible with the relative standard deviation (R.S.D.) of the mean of 2.1% obtained by replicate measurement (*n=15*) of 4.0 mg l^{-1} fluoride using the same bioelectrode (Table 3.8). Five-independently made bioelectrodes showed acceptable reproducibility with a R.S.D. of 5.0% (Table 3.9).

Table 3.7 Current response of standard fluoride

Fluoride concentration (mg l^{-1})	**Current (μA)**			**Mean (μA)**	**SD**
	1	**2**	**3**		
0.0	0.000	0.000	0.000	0.000	0.000
2.0	0.018	0.022	0.017	0.019	0.003
4.0	0.038	0.039	0.030	0.036	0.005
6.0	0.054	0.053	0.050	0.052	0.002
8.0	0.070	0.073	0.060	0.068	0.007
10.0	0.084	0.089	0.078	0.084	0.006
12.0	0.099	0.095	0.100	0.098	0.003
14.0	0.109	0.110	0.111	0.110	0.003
16.0	0.115	0.130	0.113	0.119	0.009
18.0	0.120	0.129	0.124	0.124	0.005
20.0	0.128	0.132	0.134	0.131	0.003

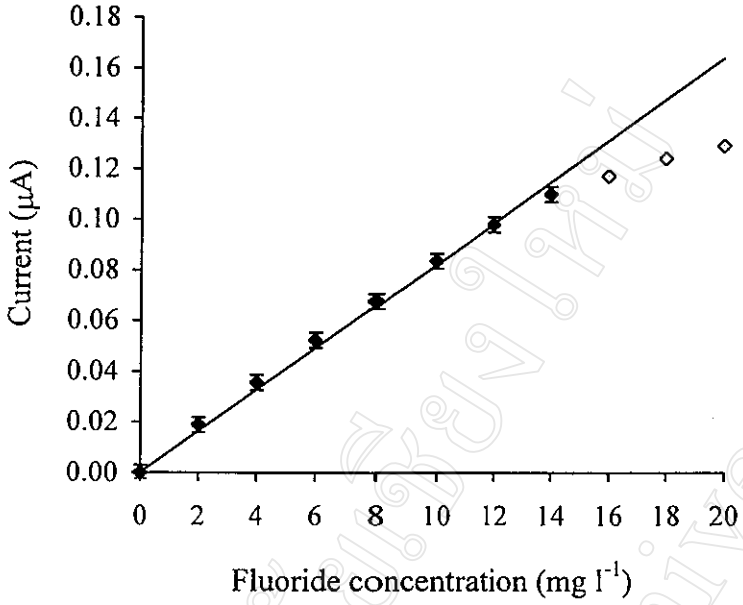

Figure 3.9 Calibration graph of fluoride

Table 3.8 Replicate measurement of fluoride using the same bioelectrode in batch system

Fluoride concentration (mg l^{-1})	**Current (μA)**	**Mean (μA)**	**SD**	**RSD (%)**
4	0.028, 0.028, 0.028, 0.028, 0.028, 0.028, 0.029, 0.028, 0.027, 0.028, 0.027, 0.028, 0.029, 0.027, 0.028	0.028	0.001	2.1

Table 3.9 Replicate measurement of fluoride using five-independently made bioelectrodes in batch system

Fluoride concentration (mg l^{-1})	Electrode No.	Current (μA)	Mean (μA)	SD	RSD (%)
4	1	0.028, 0.028, 0.027	0.025	0.001	5.0
	2	0.027, 0.026, 0.026			
	3	0.025, 0.025, 0.025			
	4	0.025, 0.025, 0.025			
	5	0.025, 0.024, 0.024			

3.1.3.2 Kinetic parameters in batch system

The apparent Michaelis-Menten constant K_m^{app}, and the maximum current density of H_2O_2 bioelectrode can be determined from the electrochemical Eadie-Hofstee form of the Michaelis-Menten equation. [67, 68]

$$I = I_{max} - K_m^{app} \left(\frac{I}{C} \right) \qquad (3.2)$$

where I is the steady-state current, I_{max} is the maximum current measured under condition of enzyme saturation and C is H_2O_2 concentration. The current response of H_2O_2 and current / [H_2O_2] value are shown in Table 3.10. The plot is graphically illustrated in Figure 3.10. The K_m^{app} and I_{max} values for the bioelectrode were found to be 0.26 mM and 1.29 μA, respectively. In comparison with previously reported H_2O_2 bioelectrode [30], K_m^{app} for the proposed bioelectrode (0.26 mM) is lower than that reported (0.41 mM).

Table 3.10 Current response of standard H_2O_2 and $\frac{\text{current}}{[H_2O_2]}$

H_2O_2 (mM)	Current (μA)*	$\frac{\text{Current}}{[H_2O_2]}$, (μA mM^{-1})
0.00	0.000	0.000
0.01	0.048	4.833
0.02	0.093	4.650
0.03	0.134	4.467
0.04	0.173	4.325
0.05	0.209	4.180
0.06	0.244	4.067
0.07	0.276	3.948
0.08	0.306	3.833
0.09	0.334	3.711
0.10	0.361	3.610
0.11	0.386	3.512

* = Mean of triplicate

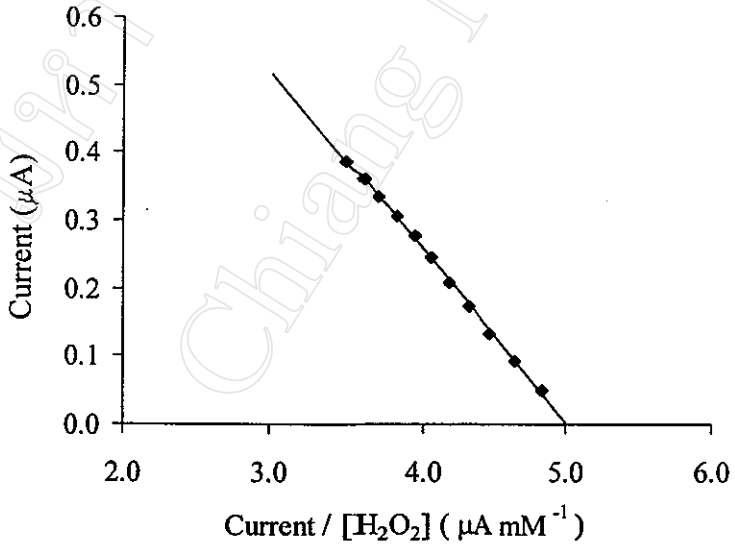

Figure 3.10 Eadie-Hofstee plot for the H_2O_2 bioelectrode

[7% (w/w) asparagus tissue and 6% w/w ferrocene); 10.0 ml of 0.05 M NaH_2PO_4-NaOH buffer (pH 5.0); applied potential, -0.05 V (*vs.* Ag/AgCl)]

The reversibility of the bioelectrode to fluoride inhibition was investigated. The concentration of H_2O_2 was measured at different concentrations of fluoride using peroxidase bioelectrode. The current response of H_2O_2 at different concentrations of fluoride is shown in Table 3.11. Lineweaver-Burk plot for series of different fluoride concentrations was plotted between $\frac{1}{\text{current}}$ and $\frac{1}{[H_2O_2]}$ according to the following equation [73].

$$\frac{1}{I} = \left(\frac{K'_m}{I_{max}}\right)\frac{1}{S} + \frac{1}{I_{max}} \qquad (3.3)$$

where K'_m is the apparent Michaelis-Menten constant in the presence of and initial concentration $[I_0]$ of the competitive inhibitor and S is the H_2O_2 concentration. The x-axis intercept corresponds to (K'_m / I_{max}) whereas the y-axis intercept corresponds to $1/I_{max}$. The values of 1 / $[H_2O_2]$ and 1/current at different concentrations of fluoride for Lineweaver-Burk plot are presented in Table 3.12. The plot is depicted in Figure 3.11. According to competitive inhibition kinetics, the apparent Michaelis constant value of the bioelectrode with the inhibitor (K'_m) is $K_m\{1+[\text{Inhibitor}] / K_i\}$ times (K_i = inhibitor constant) that without any inhibitor, while the maximum current response for substrate concentration value (I_{max}) not change at all. From Figure 3.11, it can be seen the K'_m value increases with increasing fluoride concentrations. The I_{max} values remain the same at different inhibitor concentrations. These results verified the competitive inhibition mechanism. In addition, the inhibitor and the substrate are in competition for the same binding site on the enzyme. If the inhibitor concentration is low or it held in an unsuitable position with respect to the catalytic site of the enzyme or to other potential substrates, for a reaction to take place and the inhibitor must dissociate from the enzyme and be replaced by a molecule of substrate. The degree of inhibition decreases as the substrate concentration increases. Competitive inhibition is therefore completely removed in the presence of an excess of substrate [73].

Table 3.11 Current response of H_2O_2 at different concentrations of fluoride

$\frac{1}{[H_2O_2]}$, (mM)	Current (μA) at fluoride concentration (mg l^{-1})*					
	0.0	4.0	8.0	12.0	16.0	20.0
0.05	0.43	0.398	0.379	0.365	0.352	0.341
0.10	0.59	0.561	0.537	0.520	0.505	0.492
0.20	0.75	0.717	0.695	0.676	0.662	0.646
0.30	0.77	0.746	0.732	0.720	0.709	0.698
0.40	0.83	0.814	0.798	0.785	0.776	0.766
0.50	0.83	0.808	0.795	0.783	0.774	0.768

* = Mean of triplicate

Table 3.12 $\frac{1}{[H_2O_2]}$ and $\frac{1}{current}$ at different concentration of fluoride for Lineweaver-Burk plot

$\frac{1}{[H_2O_2]}$, (mM)	$\frac{1}{current}$, (μA^{-1}) at fluoride concentration (mg l^{-1})*					
	0.0	4.0	8.0	12.0	16.0	20.0
20.0	2.342	2.510	2.639	2.737	2.838	2.930
10.0	1.686	1.783	1.861	1.924	1.979	2.033
5.0	1.399	1.395	1.481	1.489	1.511	1.547
3.3	1.302	1.340	1.339	1.389	1.411	1.432
2.5	1.227	1.250	1.282	1.273	1.288	1.306
2.0	1.204	1.220	1.236	1.235	1.235	1.235

* = Mean of triplicate

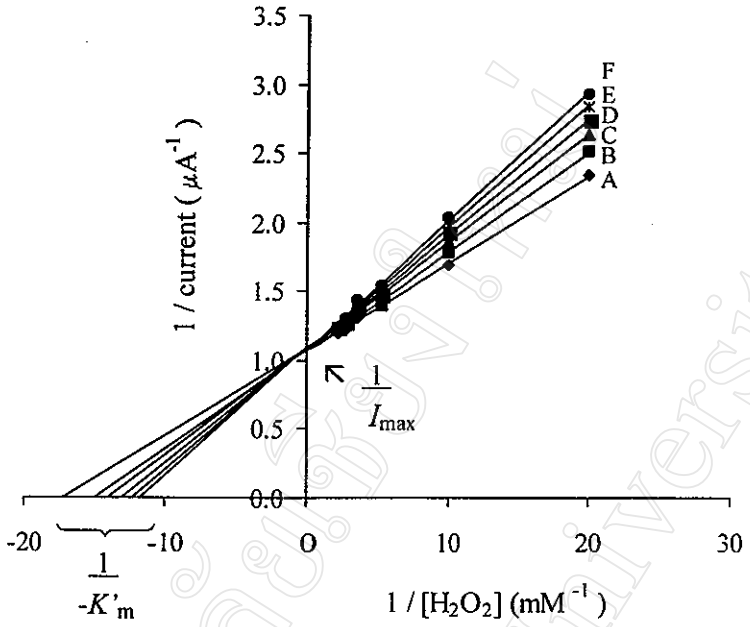

Figure 3.11 Lineweaver-Burk plot for fluoride inhibitor on the bioelectrode [with a H_2O_2 range of 0.05-5.0 mM at (A) 0.0; (B) 4.0; (C) 8.0; (D) 16.0; (E) and (F) 20 mg l^{-1} fluoride]

3.1.3.3 Response time of the bioelectrode in batch system

The response time (t_{90}), (defined as the time taken to reach 90% of the steady-state response), of the proposed bioelectrode for fluoride was 1 min as shown in Figure 3.12. Such a rapid response is the result of rapid establishment of a steady state due to low diffusion barriers.

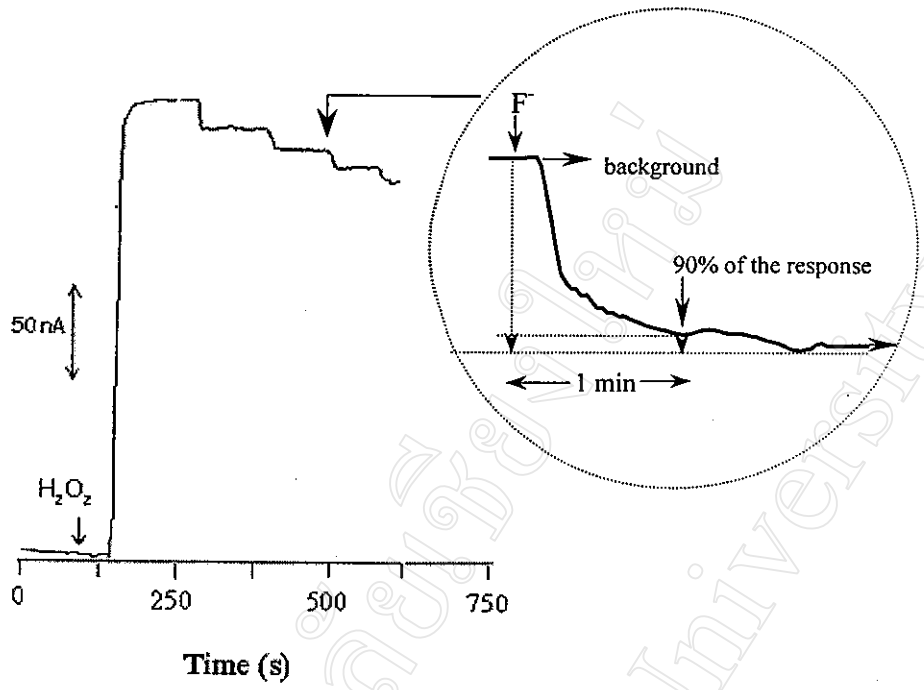

Figure 3.12 The response time (t_{90}) of 4 mg l^{-1} fluoride

3.1.3.4 Stability of the bioelectrode in batch system

The stability of the fluoride bioelectrode under the proposed storage conditions in this study was investigated (Figure 3.13). It was found that the response of the bioelectrode decreased with time. Thus, after 12 days, the bioelectrode sensitivity decreased to 50% of its original value. The loss of bioelectrode sensitivity is, therefore, ascribed to the leaching of ferrocene from the electrode and loss of the enzyme activity [30].

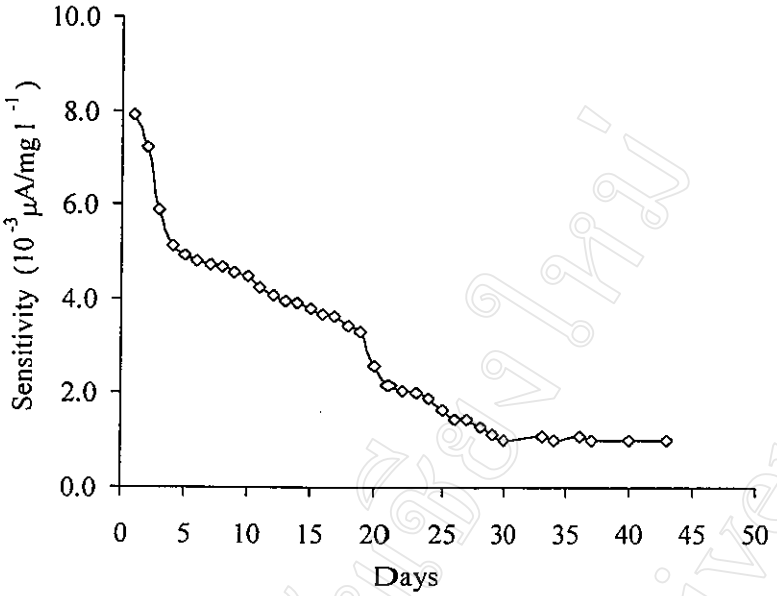

Figure 3.13 Stability of fluoride bioelectrode

[kept in phosphate buffer (pH 7.0) at 4°C (7% w/w asparagus tissue and 6% w/w ferrocene) in a 10.0 ml of 0.05 M NaH_2PO_4-NaOH buffer (pH 5.0) containing 0.1 mM H_2O_2; applied potential, -0.05 V (*vs.*Ag/AgCl)]

3.1.3.5 Interference studies in batch system

A total of 26 substances were used to evaluate the selectivity of the bioelectrode. The current obtained for each potential interference at 50 mg l^{-1} (except as otherwise stated) in the presence of fluoride at a concentration of 5 mg l^{-1} was used as an indicator for bioelectrode selectivity in comparison with the fluoride reading alone. These results are listed in Table 3.13. It is evident that the bioelectrode is quite selective for fluoride. Of these substances studied, only cyanide, sulphite, ascorbic acid, catechol (each 5 mg l^{-1}) interfered to a significant extent. As for most amperometric sensors, electroactive species such as ascorbic acid can easily be oxidized at low potential. The increase in the bioelectrode response to fluoride in the presence of cyanide and sulphite indicates that these are inhibitors of peroxidase [74-76].

Table 3.13 Possible interferences tested with the fluoride bioelectrode

Substances	Concentration (mg l^{-1})	Current ratios [a, *]
Cyanide	5	4.76
Sulphite	5	4.05
Ascorbic acid	5	1.38
Catechol	5	0.49
Oxalic acid	50	1.51
Histidine	50	1.32
Thiosulphate	50	1.16
Glycine	50	1.15
Arginine	50	1.14
Glucose	50	1.06
Starch	50	1.02
Chloride	50	1.00
Lead(II)	50	0.22
Cadmium(II)	50	0.34
Manganese(II)	50	0.37
Nickel(II)	50	0.54
Magnesium(II)	50	0.76
Nitrite	50	0.81
Cobalt(II)	50	0.83
Glutamic acid	50	0.84
Phenol	50	0.84
Nitrate	50	0.86
Sulphate	50	0.94
Tyrosine	50	0.94
Fructose	50	0.95
Zinc(II)	50	0.98

[a] Ratio of currents for mixture of 5 mg l^{-1} fluoride and substances compared with 5 mg l^{-1} fluoride alone.
* = Mean of triplicate

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3.1.3.6 Percentage recoveries in batch system

The accuracy of the bioelectrode response was evaluated by determining the recoveries of fluoride after standard addition of various concentrations of fluoride ranging from 2.0 to 5.0 mg l^{-1} to fluoride tablet sample solutions (2 mg l^{-1}). The bioelectrode shows satisfactory results with an average recovery of 97.0% (Table 3.14).

Table 3.14 Recoveries of fluoride added to fluoride tablet samples

Sample No.	Fluoride standard solution added (mg l^{-1})	Fluoride found (mg l^{-1})*	%Recovery
1	2.0	1.9	95.0
	3.0	2.9	96.7
	4.0	3.9	97.5
	5.0	4.9	98.0
2	2.0	1.9	95.0
	3.0	2.9	96.7
	4.0	3.9	97.5
	5.0	4.9	98.0
			Mean = 97.0

* = Mean of triplicate

3.1.4 Determination of fluoride content in commercial fluoride tablets

The bioelectrode was evaluated for the determination of fluoride in commercial tablet formulation. In all, 12 fluoride tablet samples selected at random were accurately weighed and calculated for an average tablet weight. The tablets were ground to fine powder followed by passing through a 60 mesh sieve. The drug powder equivalent to an average weighed tablet was taken and accurately weighed. This fluoride drug powder was completely dispersed in a conical flask that contained deionized water by heating on steam bath and shaking intermittently. The solution (40 ml) was allowed to room temperature, transferred into a 50 ml volumetric flask (after filtration) and diluted to the mark with de-ionized water. The solution was stored in a plastic bottle. The sample solutions were analyzed by the proposed method (Table 3.15) and the results were compared with those obtained by potentiometric measurement with fluoride selective electrode [65]. The potentiometric measurements were performed by using a standard addition method (appendix A). A comparison of the results is summarized in Table 3.16. A satisfactory agreement between the results was found with a range of mean relative difference of –3.4 to –4.2%. Evaluation of the proposed method was also carried out by comparison the results obtained with those obtained by potentiometric measurement with fluoride selective electrode by using t-test [71]. There are 10 degree of freedom so the critical value of t-test is 2.23 at the confidence interval of 95%. The observed value of t-test (0.06) is less than the critical value so the both methods were in good agreement (Table 3.17).

Table 3.15 Determination of fluoride in fluoride tablet samples by the proposed method

Sample No.	Fluoride concentration ($mg\ l^{-1}$)	Mean ($mg\ l^{-1}$)	SD
1	0.25, 0.25, 0.24	0.24	0.004
2	0.96, 0.94, 0.94	0.94	0.008

Table 3.16 Determination of fluoride in fluoride tablet samples by the proposed method and fluoride ion selective electrode (ISE) using a standard addition method [65]

Samples	Amount of fluoride (mg per tablet)			Relative difference(%)[a]
	Labeled	Proposed method [(a)]	ISE [(b)]	
Zymafluor ®, *Reg.No.1C1213/28* [b]	0.25	0.24	0.25	-4.2
Zymafluor ® , *Reg.No.1C1010/28* [b]	1.00	0.94	0.97	-3.2

[a] $[(a-b)/a] \times 100$

[b] Novartis Consumer Health SA Nyon, Switzerland.

Table 3.17 Calculation for t-test (in batch system)

Methods	**Amount of fluoride (mg per tablet)**	**Mean**	**SD**	**Degree of freedom**	**Observed value**	**Critical value**
ISE	0.25, 0.24, 0.25, 0.97, 0.98, 0.97	0.61	0.40	10	0.06	2.23
Proposed method	0.25, 0.25, 0.24, 0.96, 0.94, 0.94	0.60	0.38			

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3.2 Sunflower based amperometric biosensors for glycolic acid determination incorporating with flow injection system

In this section, a ferrocene-modified carbon paste bioelectrode based on sunflower leaf tissue was developed. The proposed plant tissue bioelectrode was successfully used in conjunction with the home-made flow injection (FI) system with amperometric detection for glycolic acid determination in human urine samples. The optimum conditions for such a method were established. Validation of the proposed FI method was also performed by comparative determination of glycolic acid by HPLC using t-test.

3.2.1 Cyclic voltammetry

In order to evaluate the performance of the proposed plant tissue bioelectrode, cyclic voltammograms of the electrode; (A) plain carbon paste electrode; (B) ferrocene (5%w/w)-modified electrode; (C) sunflower leaf tissue (16%w/w) and ferrocene (5%w/w)- modified carbon paste bioelectrode; (D) in the presence of $1x10^{-2}$ M glycolic acid [0.05 M NaH_2PO_4-NaOH buffer (pH 8.0); scan rate, 0.03 V s^{-1} were investigated.

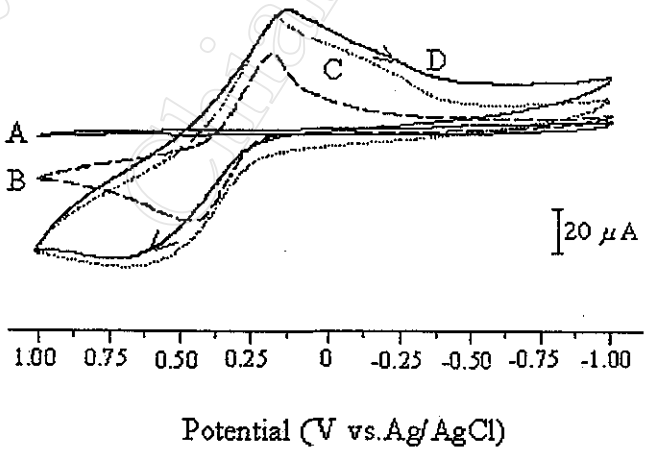

Figure 3.14 Cyclic voltammograms for glycolic acid

Figure 3.14 shows cyclic voltammograms obtained with a plain carbon paste electrode (A) and a ferrocene-modified carbon paste electrode (B). It can be seen that no redox wave was observed for a plain carbon paste electrode, whereas well-defined cathodic and anodic waves appeared at ferrocene-modified carbon paste electrode. Accordingly, the wave appearing at the latter electrode was ascribed to the electrochemical redox reaction of ferrocene molecules that had been mixed in the carbon paste electrode. The addition of sunflower leaf (*Helianthus annuus L.*) as a source of peroxidase and glycolate oxidase [77] in the ferrocene-modified paste showed little effect on the redox wave peak potential except that a larger current was observed, indicating an enhanced diffusional flux (C). The larger current obtained for the tissue-modified carbon paste compared with those of the ferrocene-modified carbon paste electrode indicated that the former electrode was much more hydrophobic than the latter, thus allowing a large surface exposure. The voltammetry of the proposed bioelectrode in the presence of glycolic acid (D) clearly exhibits an increase in the reduction current of the ferricinium ion and a decrease in the oxidation current of ferrocene. The bioelectrode mechanism can be summarized as shown in Figure 3.15.

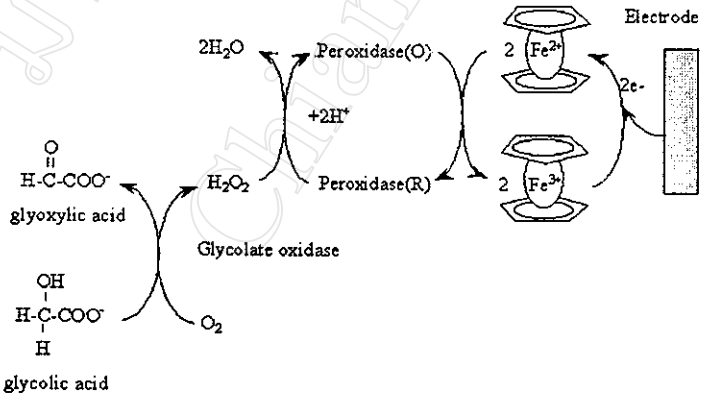

Figure 3.15 Reaction sequence of glycolic acid within the sunflower leaf tissue- based ferrocene mediated bioelectrodes [44]

3.2.2 Optimization of experimental variables in FIA system

3.2.2.1 Effect of applied potential in FIA system

The effect of applied potential on the proposed bioelectrode response to 5 mM glycolic acid is shown in Table 3.18 and Figure 3.16. It was found that the reduction current was decrease from 0.281 to 0.043 μA on changing the potential from -0.15 to +0.15 V (*vs.* Ag/AgCl). At –0.15 V the cathodic current kept increasing even without injecting glycolic acid and the noise increased dramatically. This behavior was ascribed to the reduction of dissolved oxygen at this potential. The potential of 0.00 V was selected for the subsequent experiments as the best compromise between the resulting signal and the noise level.

Table 3.18 Effect of applied potential on glycolic acid bioelectrode in FIA system

Potential (V)	Peak height (μA)			Mean (μA)	SD
	1	2	3		
-0.15	0.281	0.276	0.286	0.281	0.005
-0.10	0.253	0.275	0.287	0.272	0.017
-0.05	0.259	0.251	0.271	0.260	0.010
0.00	0.227	0.204	0.204	0.212	0.013
+0.05	0.151	0.156	0.164	0.156	0.007
+0.10	0.088	0.087	0.085	0.086	0.002
+0.15	0.049	0.040	0.041	0.043	0.005

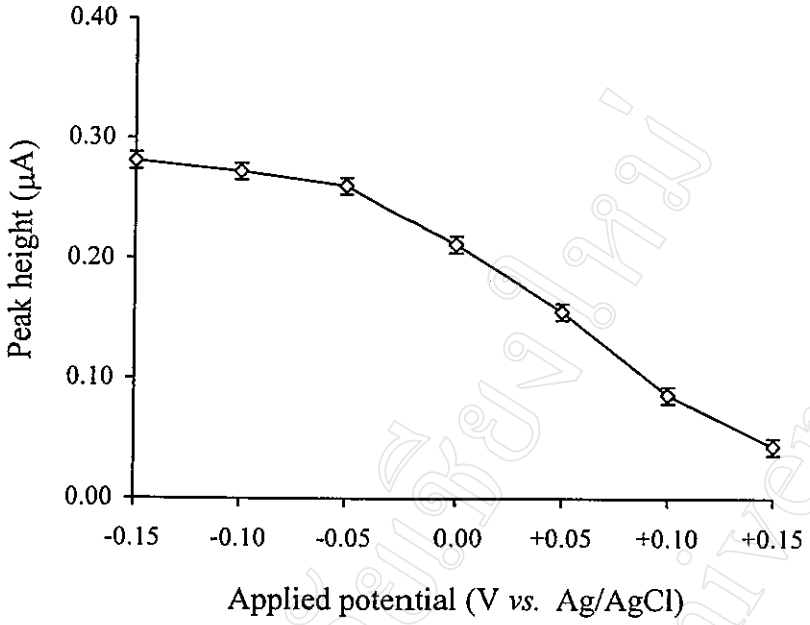

Figure 3.16 Effect of applied potential on glycolic acid bioelectrode in FIA system [50 μl of 5 mM glycolic acid; 20% (w/w) sunflower leaf tissue and 5% (w/w) ferrocene; carrier, 0.05 M NaH_2PO_4-NaOH buffer (pH 7.5); flow rate, 0.4 ml min^{-1}]

3.2.2.2 Effect of pH in FIA system

The pH dependence of the bioelectrode was studied at 5 mM glycolic acid over the pH range 4.0 to 10.0. The results were illustrated in Table 3.19 and Figure 3.17 reveals that the bioelectrode showed a maximum sensitivity of the response at pH 8.0. An optimum pH value of 8.3 has been reported for the spinach glycolate oxidase [78] and pH 7.5 has been reported in previous work [44] which nearly equal to an optimum pH value of sunflower glycolate oxidase from this work. Based on the results obtained, the phosphate buffer solution pH 8.0 was selected for subsequent studies.

Table 3.19 Influence of pH value on the glycolic acid bioelectrode in FIA system

pH	Peak height (μA)			Mean (μA)	SD
	1	2	3		
5.0	0.104	0.099	0.110	0.104	0.006
6.0	0.142	0.101	0.115	0.119	0.021
7.0	0.216	0.221	0.210	0.216	0.006
7.5	0.203	0.234	0.240	0.226	0.020
8.0	0.245	0.250	0.231	0.242	0.010
8.5	0.228	0.211	0.222	0.220	0.009
9.0	0.212	0.223	0.197	0.210	0.013
10.0	0.193	0.181	0.157	0.177	0.018

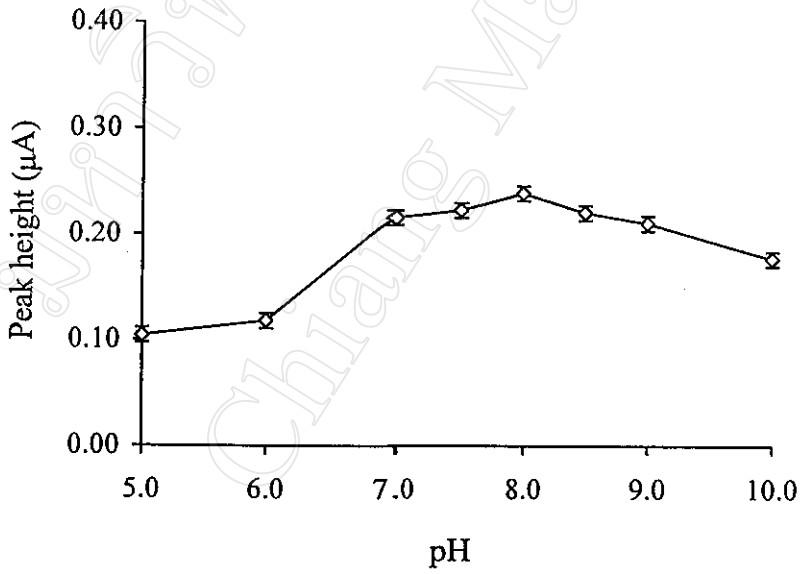

Figure 3.17 Influence of pH value on the glycolic acid bioelectrode in FIA system [20% (w/w) sunflower leaf tissue and 5% (w/w) ferrocene; 50 μl of 5 mM glycolic acid; 0.05 M NaH_2PO_4-NaOH buffer; flow rate, 0.4 ml min^{-1}; applied potential, 0.00 V (*vs.* Ag/AgCl)]

3.2.2.3 Effect of buffer types in FIA system

The effect of 5 buffer types, NaH_2PO_4-NaOH, Na_2HPO_4-NaH_2PO_4, boric-Na_2HPO_4, tartaric-NaOH and tris-HCl on the responses of the proposed bioelectrode were investigated and the results were illustrated in Table 3.20 and Figure 3.18. It can be seen that the NaH_2PO_4-NaOH buffer (pH 8.0) is the most efficient working buffer because it gives the highest response among the 5 types of buffers studied. Hence, it was employed for the further studies.

Table 3.20 Effect of buffer type on glycolic acid bioelectrode in FIA system

Buffers	Peak height (μA)			Mean (μA)	SD
	1	2	3		
NaH_2PO_4-NaOH	0.234	0.247	0.240	0.240	0.007
Na_2HPO_4-NaH_2PO_4	0.127	0.149	0.144	0.140	0.012
Boric-Na_2HPO_4	0.155	0.138	0.130	0.141	0.013
Tartaric-NaOH	0.210	0.217	0.240	0.222	0.016
Tris-HCl	0.057	0.053	0.048	0.053	0.005

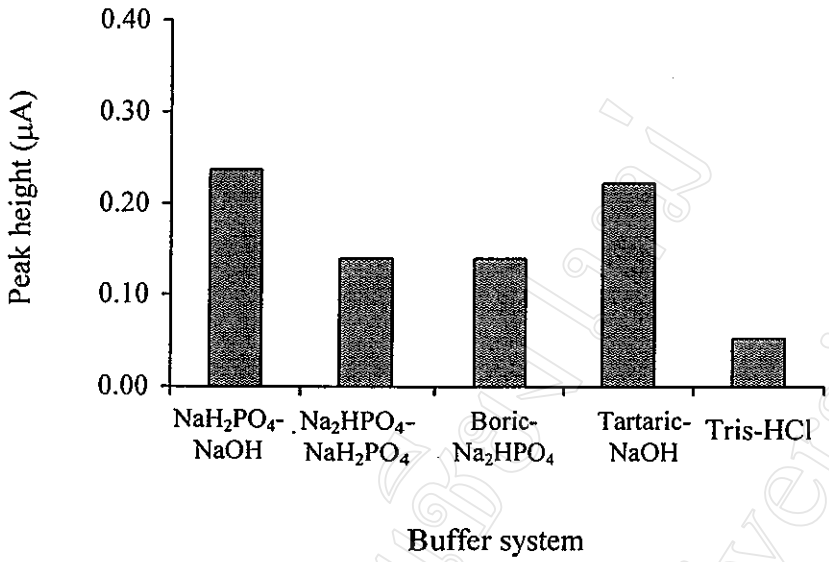

Figure 3.18 Effect of buffer type on glycolic acid bioelectrode in FIA system [20% (w/w) sunflower leaf tissue and 5% (w/w) ferrocene; 50 μl of 5 mM glycolic acid; applied potential, 0.00 V (*vs.* Ag/AgCl), pH 8.0; flow rate, 0.4 ml min^{-1}]

3.2.2.4 Effect of tissue loading in FIA system

The tissue loading has a profound effect upon the response of bioelectrode to glycolic acid. As would be expected, increasing the tissue composition of the bioelectrode from 10% to 20% (w/w) resulted in an increase in the bioelectrode response (Table 3.21 and Figure 3.19), reflecting the increase in the biocatalytic activity. However, the noise level also gradually increased with the increase in tissue content. Increasing the tissue composition beyond 20%(w/w) caused the lower responses. This behavior is ascribed to the lowering of the electrical conductivity due to the reduction in graphite loading [79]. A tissue loading of 20%(w/w) was selected for the next experiments.

Table 3.21 Effect of sunflower leaf tissue loading on glycolic acid bioelectrode in FIA system

Tissue loading (%w/w)	Peak height (μA)			Mean (μA)	SD
	1	2	3		
0.0	0.000	0.000	0.000	0.000	0.000
10.0	0.111	0.114	0.114	0.113	0.002
15.0	0.185	0.187	0.188	0.186	0.002
17.5	0.220	0.231	0.229	0.227	0.006
20.0	0.224	0.247	0.240	0.237	0.012
22.5	0.231	0.227	0.224	0.227	0.004
25.0	0.177	0.188	0.205	0.190	0.014
30.0	0.161	0.158	0.167	0.162	0.005

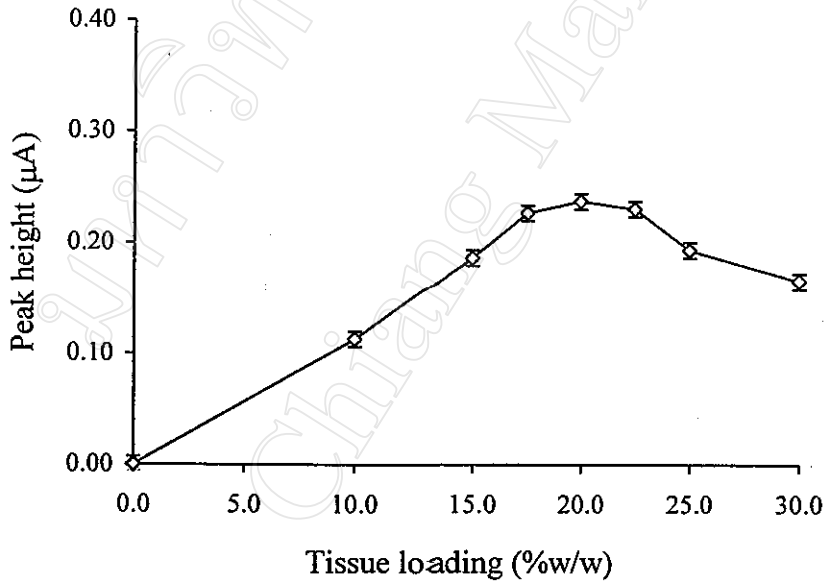

Figure 3.19 Effect of sunflower leaf tissue loading on glycolic acid bioelectrode in FIA system [5% (w/w) ferrocene mediator; 50 μl of 5 mM glycolic acid; 0.05 M NaH_2PO_4-NaOH buffer (pH 8.0); flow rate, 0.4 ml min^{-1}; applied potential, 0.00 V (*vs.* Ag/AgCl)]

3.2.2.5 Effect of mediator loading in FIA system

Table 3.22 and Figure 3.20 show the influence of mediator loading on the bioelectrode response. It can be seen that signals increasing substantially with increasing in the mediator loading from 2 to 5% (w/w). Further increase in the mediator loading above 5% resulted in a decrease in the bioelectrode response. Such a decrease in response of the bioelectrode when a larger amount of mediators is a typical behavior of a mediator-based sensor [80] and is ascribed to the lowering of the electrical conductivity due to the reduction in graphite loading [81, 82]. Based on these results, the bioelectrode with 5%(w/w) ferrocene was employed.

Table 3.22 Effect of mediator loading on the glycolic acid bioelectrode in FIA system

Mediator loading (%w/w)	Peak height (μA)			Mean (μA)	SD
	1	2	3		
0.0	0.021	0.022	0.023	0.022	0.001
2.0	0.120	0.112	0.112	0.115	0.005
3.0	0.172	0.167	0.176	0.171	0.005
4.0	0.239	0.243	0.228	0.237	0.008
5.0	0.241	0.242	0.244	0.242	0.002
6.0	0.221	0.220	0.220	0.220	0.001
7.0	0.199	0.199	0.212	0.203	0.008
9.0	0.177	0.185	0.175	0.180	0.005
11.0	0.160	0.165	0.164	0.163	0.003

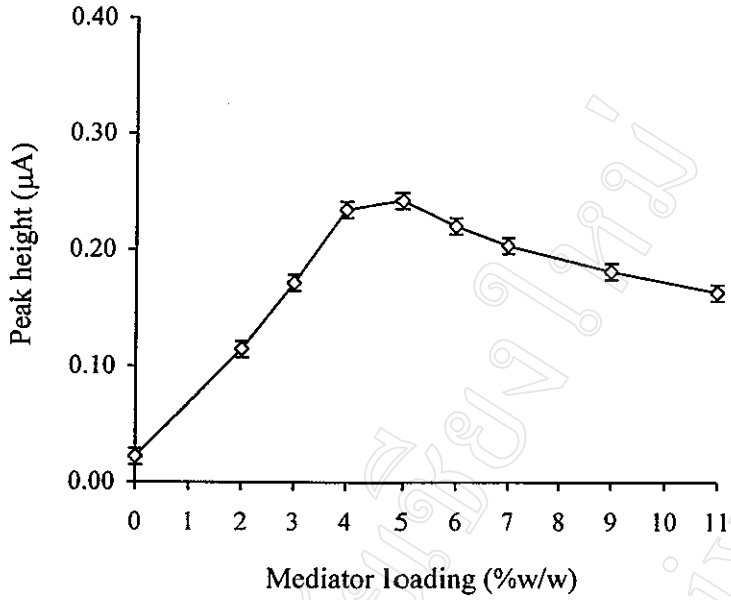

Figure 3.20 Effect of mediator loading on glycolic acid bioelectrode in FIA system [20% (w/w) sunflower leaf tissue; 50 μl of 5 mM glycolic acid; 0.05 M NaH_2PO_4-NaOH buffer (pH 8.0); flow rate, 0.4 ml min^{-1}; applied potential, 0.00 V (*vs.* Ag/AgCl)]

3.2.2.6 Effect of carrier flow rate in FIA system

The carrier flow rate dependence of the bioelectrode was pursued by varying the flow rate from 0.2 to 1.8 ml min^{-1}. The results are illustrated in Table 3.23 and Figure 3.21. It was found that the maximum response was obtained at the lowest flow rate tested (0.2 ml min^{-1}), then decreased when the flow rate was greater than 0.2 ml min^{-1}. The fast decrease in bioelectrode response with flow rate is due to a reduced residence time at the bioelectrode. Diffusion within electrode surface also plays an important role in the mass transfer because less substrate reaches the electrode surface at shorter response time [80]. However, the maximum response time is obtained at the flow rate of 0.2 ml min^{-1}. The flow rate was set at 0.3 ml min^{-1} for compromise between the bioelectrode response and response time.

Table 3.23 The influence of carrier flow rate on glycolic acid bioelectrode in FIA system

Flow rate (ml min^{-1})	Peak height (μA)			Mean (μA)	SD
	1	2	3		
0.2	0.307	0.310	0.308	0.308	0.002
0.3	0.300	0.299	0.299	0.299	0.001
0.4	0.259	0.248	0.255	0.251	0.006
0.6	0.220	0.225	0.227	0.224	0.004
0.8	0.192	0.190	0.198	0.193	0.004
1.0	0.178	0.178	0.179	0.178	0.001
1.4	0.174	0.172	0.169	0.171	0.003
1.8	0.160	0.169	0.163	0.164	0.005

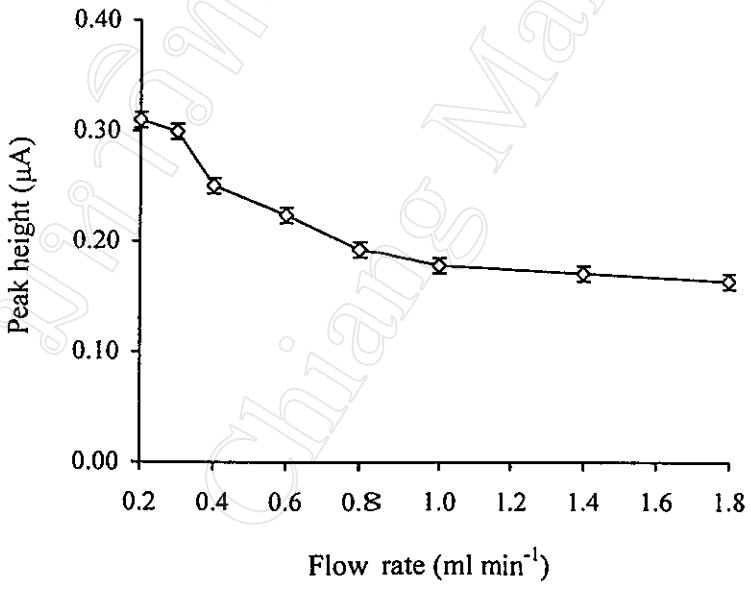

Figure 3.21 The influence of carrier flow rate on glycolic acid bioelectrode in FIA system [20%(w/w) sunflower leaf tissue and 5%(w/w) ferrocene; 50 μl of 5 mM glycolic acid; 0.05 M NaH_2PO_4-NaOH buffer (pH 8.0); applied potential, 0.00 V (*vs.* Ag/AgCl)]

3.2.2.7 Effect of injection volume in FIA system

Table 3.24 and Figure 3.22 show the dependent of the response on the injection volume. It was found that the bioelectrode response increased when the injection volume was increased from 50 to 150 µl and then started to level off. Based on the results, the sample volume of 150 µl was employed it results the highest bioelectrode response with the minimum of sample volume.

Table 3.24 Effect of injection volume on glycolic acid bioelectrode in FIA system

Injection volume (µl)	Peak height (µA)			Mean (µA)	SD
	1	2	3		
50	0.300	0.302	0.293	0.298	0.005
100	0.396	0.379	0.391	0.389	0.009
150	0.442	0.434	0.448	0.441	0.007
200	0.435	0.436	0.432	0.434	0.002
250	0.430	0.439	0.421	0.430	0.009
300	0.431	0.431	0.424	0.428	0.004

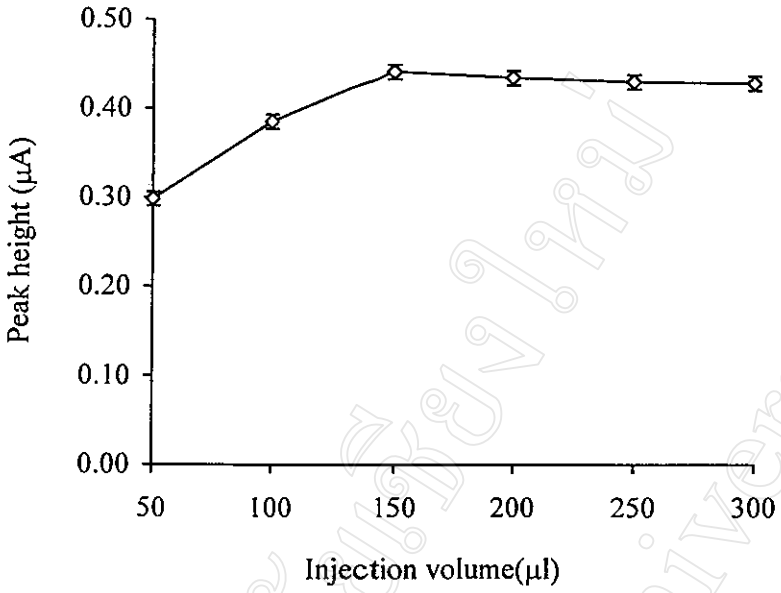

Figure 3.22 Effect of injection volume on glycolic acid bioelectrode in FIA system [20%(w/w) sunflower leaf tissue and 5%(w/w) ferrocene; 5 mM glycolic acid; 0.05 M NaH_2PO_4-NaOH buffer (pH 8.0); flow rate, 0.3 ml min^{-1}; applied ' potential, 0.00 V (*vs.* Ag/AgCl)]

3.2.2.8 Summary of the optimum conditions in FIA system

The experimental variables of sunflower based amperometric biosensor in flow injection system for glycolic acid determination had been optimized. The variables, their ranges studied and optimum conditions are shown in Table 3.25.

Table 3.25 Experimental variables, their ranges studied and optimum values for glycolic acid bioelectrode in FIA system

Experimental variables	**Range studied**	**Value chosen**
Applied potential (V) (*vs.* Ag/AgCl)	-0.15 to +0.15	0.0
pH	5.0 - 10.0	8.0
Buffer system	NaH_2PO_4-NaOH, Na_2HPO_4-NaH_2PO_4, boric-Na_2HPO_4, tartaric-NaOH and tris-HCl	NaH_2PO_4-NaOH
Tissue loading (%, w/w)	0 – 30	20
Ferrocene loading (%, w/w)	0 – 11	5
Carrier flow rate (ml min^{-1})	0.2 - 1.8	0.3
Injection volume (µl)	50 - 300	150

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3.2.3 Analytical characteristics of the FI method based on the sunflower leaf tissue bioelectrode

3.2.3.1 Calibration, detection limit and reproducibility

Figure 3.23 displays typical current recording for FIA responses under the optimized experimental conditions of the resulting calibration for glycolic acid over the concentration range 0.1- 4.0 mM. It was found that the bioelectrode exhibits linearity of calibration up to 2.0 mM. The linear regression analysis current (y) *versus* glycolic acid concentration (x) yielded the regression equation as follow:

$$y = 1.92\text{x}10^{5}\ x \qquad (r = 0.995) \qquad (3.4)$$

The slope of the initial linear range is $1.92\text{x}10^{5}$ μA mM^{-1} (Table 3.26 and Figure 3.24). A detection limit (*S/N=3*) of $1\text{x}10^{-6}$ M was obtained for the bioelectrode. The replicate measurements of 1.0 mM glycolic acid for the same bioelectrode yielded 1.67% (*n=15*) relative standard deviation from the mean (Table 3.27). Five independently made bioelectrodes showed acceptable bioelectrode reproducibility with a relative standard deviation of 4.5% (Table 3.28).

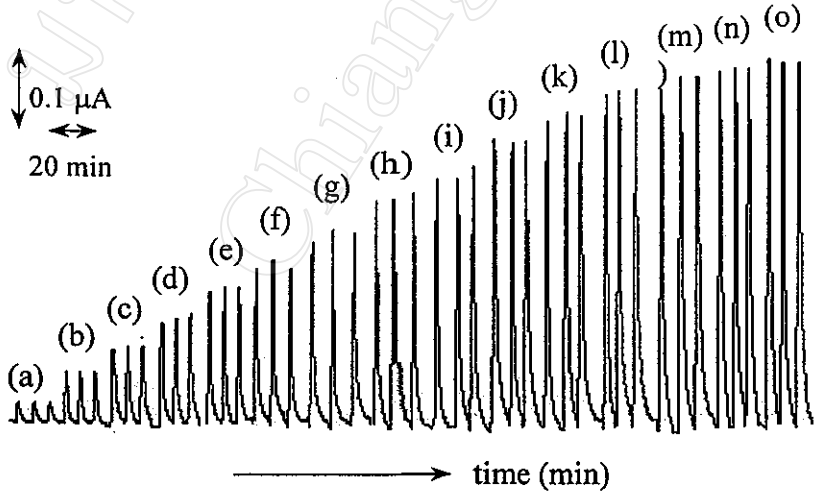

Figure 3.23 Typical response of glycolic acid in FIA system
[(a) 0.1 (b) 0.3, (c) 0.5, (d) 0.7, (e) 0.9, (f) 1.1, (g) 1.3, (k) 1.5, (i) 1.7, (j) 1.9, (k) 2.1, (l) 2.5, (m) 3.0, (n) 3.5 and (o) 4.0 mM glycolic acid]

Table 3.26 Peak heights of standard glycolic acid in FIA system

Glycolic acid concentration (mM)	Peak height (μA)			Mean (μA)	SD
	1	2	3		
0.0	0.000	0.000	0.000	0.000	0.000
0.1	0.023	0.024	0.023	0.023	0.001
0.3	0.066	0.068	0.066	0.067	0.001
0.5	0.115	0.101	0.106	0.108	0.007
0.7	0.150	0.150	0.151	0.150	0.000
0.9	0.183	0.188	0.185	0.185	0.003
1.1	0.222	0.232	0.221	0.225	0.006
1.3	0.255	0.256	0.255	0.255	0.001
1.5	0.282	0.289	0.299	0.290	0.008
1.7	0.323	0.323	0.319	0.322	0.002
1.9	0.348	0.346	0.341	0.345	0.003
2.1	0.374	0.370	0.381	0.375	0.006
2.5	0.426	0.445	0.431	0.434	0.010
3.0	0.473	0.470	0.468	0.470	0.003
3.5	0.502	0.498	0.500	0.500	0.002
4.0	0.517	0.526	0.517	0.520	0.005

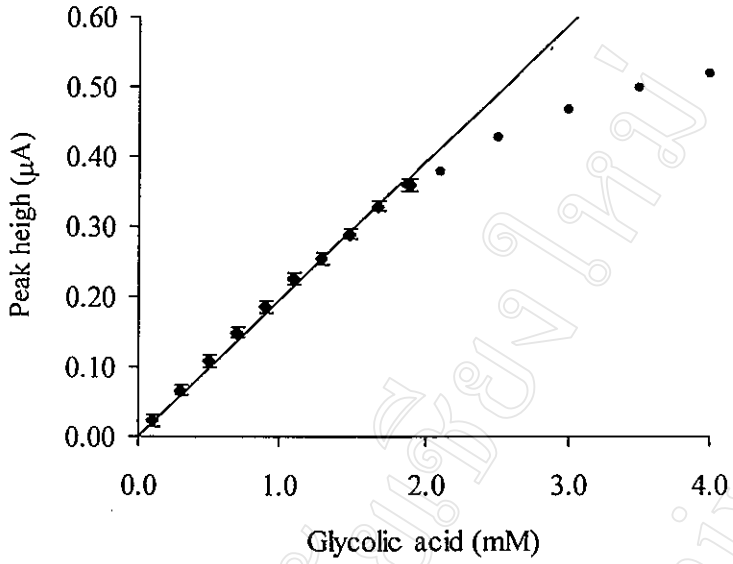

Figure 3.24 Calibration graph of glycolic acid in FIA system

Table 3.27 Replicate measurement of glycolic acid using the same bioelectrode in FIA system

Glycolic acid concentration (mM)	**Current (μA)**	**Mean (μA)**	**SD**	**RSD (%)**
1.0	0.151, 0.149, 0.152, 0.149, 0.150, 0.145, 0.147, 0.150, 0.147, 0.145, 0.148, 0.147, 0.148, 0.147, 0.143	0.148	0.003	1.67

Table 3.28 Replicate measurement of glycolic acid using five-independently made bioelectrode in FIA system

Glycolic acid concentration (mM)	Electrode No.	Current (μA)	Mean (μA)	SD	RSD (%)
1.0	1	0.149, 0.150, 0.150	0.146	0.007	4.5
	2	0.144, 0.137, 0.139			
	3	0.148, 0.150, 0.146			
	4	0.142, 0.132, 0.142			
	5	0.151, 0.157, 0.151			

3.2.3.2 Michaelis and Menten constant in FIA system

The apparent Michaelis-Menten constant K_m^{app} , and the maximum current density of glycolic acid sensor can be determined from the electrochemical Eadie-Hofstee form of the Michaelis-Menten equation. [67, 68]

$$I = I_{max} - K_m^{app}\left(\frac{I}{C}\right) \qquad (3.5)$$

Where I is the steady-state current, I_{max} is the maximum current measured under conditions of enzyme saturation and C is glycolic acid concentration. The current response of standard glycolic acid and current / [glycolic acid] are shown in Table 3.29. The plot is graphically illustrated in Figure 3.25. The K_m^{app} and I_{max} values for the bioelectrode were found to be 6.56 mM and 1.5μA, respectively.

Table 3.29 Current responses of standard glycolic acid and $\frac{\text{Current}}{\text{[Glycolic acid]}}$ in FIA system

Glycolic acid concentration (mM)	**Current (μA)***	$\frac{\textbf{Current}}{\textbf{[Glycolic acid]}}$**, (μA mM^{-1})**
0.1	0.024	0.235
0.3	0.067	0.223
0.5	0.108	0.216
0.7	0.150	0.214
0.9	0.185	0.206
1.1	0.225	0.205
1.3	0.255	0.196
1.5	0.290	0.193
1.7	0.322	0.189
1.9	0.345	0.182
2.1	0.375	0.179

* = Mean of triplicate

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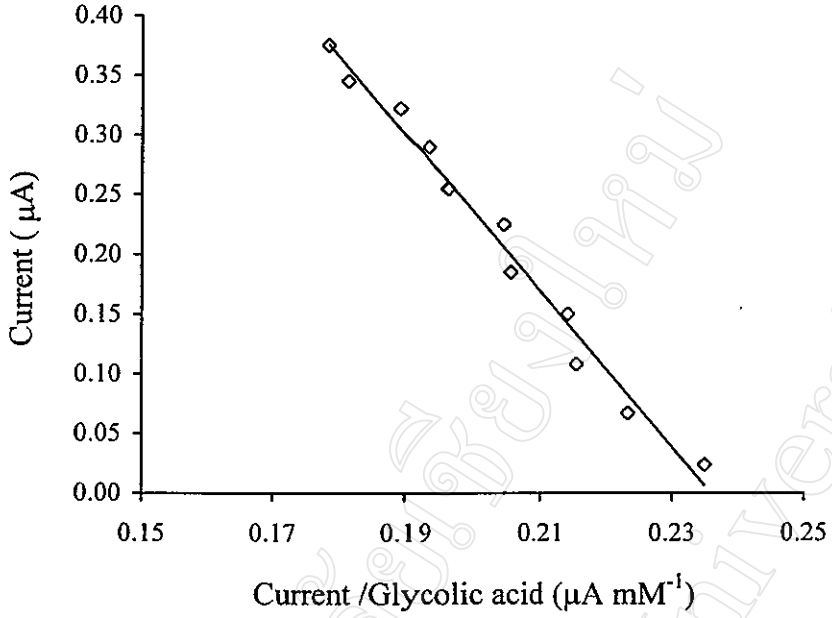

Figure 3.25 Eadie-Hofstee plot for the glycolic acid bioelectrode in FIA system [20% (w/w) sunflower leaf tissue and 5% (w/w) ferrocene; 50 μl, 5 mM glycolic acid; 0.05 M NaH_2PO_4-NaOH buffer (pH 8.0); flow rate, 0.3 ml min^{-1}; applied potential, 0.00 V (*vs.* Ag/AgCl)]

3.2.3.3 Sample throughput in FIA system

The half peak-widths were found to be 90 sec. These allow injection rates of 30 samples h^{-1} to be achieved. The high sample throughput reflects the inherent advantage of the FIA technique over the batch measurements.

3.2.3.4 Stability of the bioelectrode in FIA system

An important parameter when considering the merit of the bioelectrode in the flow system is the electrode stability. To investigate the present electrode stability, aliquots of a standard solution of glycolic acid (1.0 mM) were injected continuously into the FIA system under the optimized conditions. Figure 3.26 shows the peak currents of glycolic acid. It can be seen that the response of bioelectrode remains almost constant initially, up to 70 injections, because the enzyme is in excess. After 90 and 110 injections, the bioelectrode response decreased to 70% and 50% of the original value, respectively. The loss of bioelectrode response is, therefore, ascribed to the leaching of ferrocene from the electrode and loss of the enzyme activity.

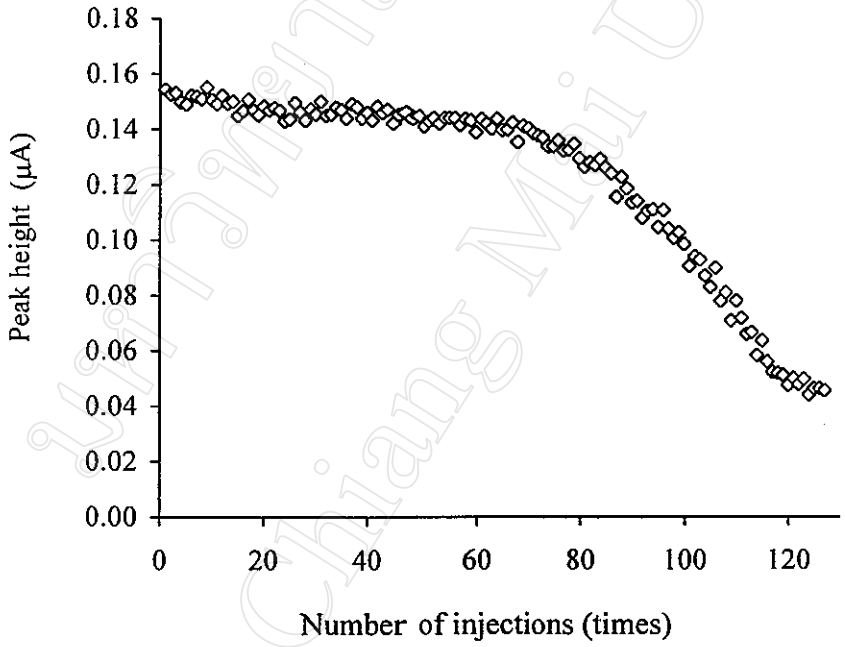

Figure 3.26 Stability of glycolic acid bioelectrode in FIA system

[20%(w/w) sunflower leaf tissue and 5%(w/w) ferrocene; 50 μl, 5 mM glycolic acid; 0.05 M NaH_2PO_4-NaOH buffer (pH 8.0); flow rate, 0.3 ml min^{-1}; applied potential, 0.00 V (*vs.* Ag/AgCl)]

3.2.3.5 Interference studies in FIA system

Fourteen possible interferences were tested to evaluate the selectivity of the bioelectrode. The current obtained for each interference at 5 mM (except as otherwise stated) in the presence of glycolic acid at a concentration 0.5 mM was used as an indicator for the bioelectrode selectivity in comparison with the glycolic acid reading alone. These results are listed in Table 3.30, showing that the bioelectrode is quite selective for glycolic acid. Of these interferences studied, only ascorbic acid (0.1 mM), uric acid (5 mM) and lactic acid (5 mM) interfered to a significant extent. As for most amperometric sensors, electroactives species such as ascorbic acid can be easily oxidized directly on the electrode surface at low potential, thus changing the enzyme-mediated current. These effects clearly occur in the present work. However, in the presence of lower concentration of ascorbic acid (0.005 mM), lactic acid (0.2 mM) and uric acid (0.2 mM), it was found that no interference effects on the glycolic acid determination were observed. The small bioelectrode response to uric acid and lactic acid implies some cross-reactivity of enzyme glycolate oxidase towards uric acid and lactic acid. This result is consistent with previous work [78, 84-85] regarding the ability of glycolate oxidase to oxidize uric acid and lactic acid.

Table 3.30 Possible interferences tested with the glycolic acid bioelectrode in FIA system

Substances	Concentration (mM)	Current ratios [a],*
Ascorbic acid	0.1	0.29
Oxalic acid	5	0.84
Tartaric acid	5	0.84
Salicylic acid	5	0.88
Glucose	5	0.90
Fructose	5	0.93
Succinic acid	5	0.95
Malonic acid	5	0.99
Maleic acid	5	1.05
Formic acid	5	1.09
Citric acid	5	1.14
Acetic acid	5	1.16
Glutamic acid	5	1.27
Uric acid	5	1.52
Lactic acid	5	1.67

[a] Current ratios for mixture of concentration of substance and 0.5 mM glycolic acid compared with 0.5 mM glycolic acid alone.
* = Mean of triplicate

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3.2.3.6 Percentage recoveries in FIA system

For determining the recoveries of glycolic acid, the accuracy of the bioelectrode has been evaluated after 0.5 and 1.0 mM were added to a 10-fold dilution of charcoal pretreated urine. Other compounds may interfere by reacting with hydrogen peroxide, ferrocene or by direct electrochemical activity at the applied voltage. The pretreatment step of urine sample with charcoal is essential and recommended in order to eliminate any ascorbic acid and other electroactive compounds contained in the samples. The samples were mixed with 300 mg of activated charcoal and the charcoal was removed by centrifugation. A 10 fold-dilution of the supernatant was then used for the glycolic acid assay [86]. The results obtained are given in Table 3.31. The bioelectrode shows satisfactory results with an average recovery of 98.7%.

Table 3.31 Recoveries of glycolic acid added to a 10 fold-dilution of pretreated urine samples in FIA system

Sample No.	Glycolic acid concentration (mM)		%Recovery
	Added	Found*	
1	0.5	0.51	102.0
	1.0	1.02	102.0
2	0.5	0.48	96.0
	1.0	1.00	100.0
3	0.5	0.48	96.0
	1.0	0.96	96.0
			Mean = 98.7

* = Mean of triplicate

3.2.4 Determination of glycolic acid in urine samples in FIA system

A 10 fold-dilution of pretreated urine samples were analyzed by the present method (Table 3.32) and the results were compared with HPLC method (appendix B). Comparison of the results obtained is summarized in Table 3.33. A satisfactory agreement between the results was found with mean relative differences in the range 0.0 – 1.0%. Moreover, the validation of the proposed FI method was also performed by comparative determination of glycolic acid by HPLC using t-test. The observed value of the proposed method is 0.14 and the critical value of t-test is 2.12 (18 degree of freedom) at the confidence interval of 95% (Table 3.34). The results obtained by the proposed method are excellent agreement with those determined by HPLC [87].

Table 3.32 Results of glycolic acid determination in 10 fold-dilution of pretreated urine samples by the proposed method (FIA system)

Samples No.	Glycolic acid concentration (mM)	Mean (mM)	SD
1	0.50, 0.49, 0.48	0.49	0.007
2	0.73, 0.72, 0.72	0.72	0.009
3	0.70, 0.73, 0.73	0.72	0.016

Table 3.33 Results of glycolic acid determination in 10 fold-dilution of pretreated urine samples by the proposed method (FIA system) and the HPLC method [87]

Samples No.	Glycolic acid concentration (mM)		Relative difference (%) [c]
	Proposed method [a, *]	HPLC method [b,*]	
1	0.49	0.49	0.0
2	0.72	0.71	1.0
3	0.72	0.71	1.0

[c] = [(a – b) / a] x 100
* = Mean of triplicate

Table 3.34 Calculation for t-test (FIA system)

Methods	Glycolic acid concentration (mM)	Mean	SD	Degree of freedom	Observed value	Critical value
Proposed method	0.50, 0.49, 0.48, 0.73, 0.72, 0.72, 0.70, 0.73, 0.73	0.65	0.12	18	0.14	2.12
HPLC	0.48, 0.50, 0.49, 0.71, 0.71, 0.71, 0.70, 0.71, 0.72	0.64	0.11			

3.3 Sunflower based amperometric biosensors for glycolic acid determination incorporating with batch injection system

In this section, the proposed sunflower based bioelectrode used in section 3.2 has been adopted to the development of the batch injection method for glycolic acid determination. Optimum condition for determination glycolic acid was investigated. The method was again applied to the determination of glycolic acid in human urine samples. Validation of the proposed method was also performed.

3.3.1 Cyclic voltammetry

Cyclic voltammograms of plain carbon paste electrode, ferrocene (5%w/w)-modified electrode, sunflower leaf tissue (16%w/w), ferrocene (5%w/w)-modified carbon paste bioelectrode and in the presence of 10 mM glycolic acid have been shown in Figure 3.14 (section 3.2.1)

3.3.2 Optimization of experimental variables in BIA system

3.3.2.1 Effect of applied potential in BIA system

The effect of applied potential on the bioelectrode response is shown in Table 3.35 and Figure 3.27. It was found that the bioelectrode sensitivity decreased as the applied potential was changed from –0.15 V to +0.15 V (*vs.* Ag/AgCl). As the potential approached 0.15 V, the noise increased dramatically. This behavior is ascribed to the electrochemical oxidation of the ferrocene contained in the paste occurring in this potential range. At –0.15 V, the cathodic current kept increasing even without injecting glycolic acid. This behavior was ascribed to the reduction of dissolved oxygen at this potential as described earlier in section 3.2.2.1. A potential of 0.00 V was selected for the subsequent of the experiments as the best compromise between the resulting signal and the noise level. This low potential is also expected to minimize possible interferences.

Table 3.35 Effect of applied potential on glycolic acid bioelectrode in BIA system

Applied potential (V)	Current (μA)* at [glycolic acid] (mM)				Sensitivity** (μA mM^{-1})
	0.1	0.3	0.5	1.0	
-0.15	0.028	0.086	0.166	0.235	0.257
-0.10	0.025	0.079	0.144	0.227	0.241
-0.05	0.037	0.088	0.149	0.207	0.231
0.00	0.027	0.082	0.126	0.210	0.222
+0.05	0.017	0.054	0.103	0.150	0.163
+0.10	0.020	0.052	0.070	0.112	0.122
+0.15	0.018	0.035	0.046	0.084	0.088

* = mean of triplicate

** = The sensitivity is defined as the slope (ΔI/[glycolic acid]) of a plot of response *versus* glycolic acid concentration.

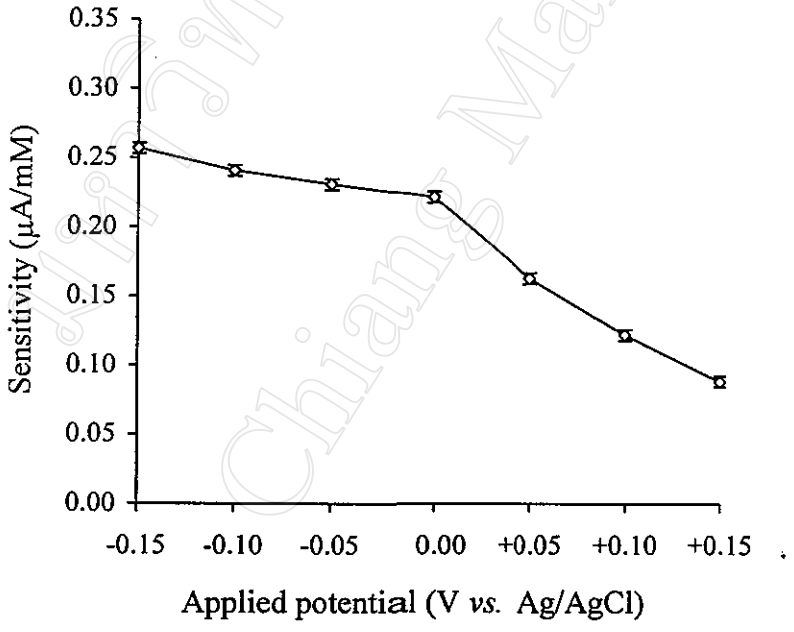

Figure 3.27 Effect of applied potential on glycolic acid bioelectrode in BIA system [30 μl of 0.1-1.0 mM glycolic acid ; 05 M NaH_2PO_4-NaOH buffer (pH 8.0); 6%(w/w) sunflower leaf tissue and 5%(w/w) ferrocene; stirring rate, 100 rpm; tip-electrode distance, 1 mm]

3.3.2.2 Effect of pH in BIA system

The pH dependence of the bioelectrode over the pH range of 5.0 to 10.0 is illustrated in Table 3.36 and Figure 3.28. The resulting peak-shaped pH profile showed maximum sensitivity of the bioelectrode response at pH 8.0. The optimum pH values of 7.5 and 8.3 for spinach glycolate oxidase had been reported in previous work [44, 78] which were nearly similar to an optimum pH value of sunflower glycolate oxidase in this work. Based on the results obtained, the phosphate buffer solution of pH 8.0 was selected for subsequent experiments.

Table 3.36 Effect of pH value on glycolic acid bioelectrode in BIA system

pH	Current (μA)* at [glycolic acid] (mM)				Sensitivity** (μA mM^{-1})
	0.1	0.3	0.5	1.0	
5.0	0.014	0.026	0.042	0.066	0.071
6.0	0.011	0.048	0.086	0.105	0.121
7.0	0.022	0.064	0.082	0.174	0.175
7.5	0.023	0.074	0.123	0.199	0.211
8.0	0.028	0.076	0.123	0.225	0.231
8.5	0.031	0.078	0.115	0.203	0.212
9.0	0.026	0.061	0.114	0.166	0.181
10.0	0.010	0.055	0.099	0.115	0.135

* = mean of triplicate

** = The sensitivity is defined as the slope (ΔI/[glycolic acid]) of a plot of response *versus* glycolic acid concentration.

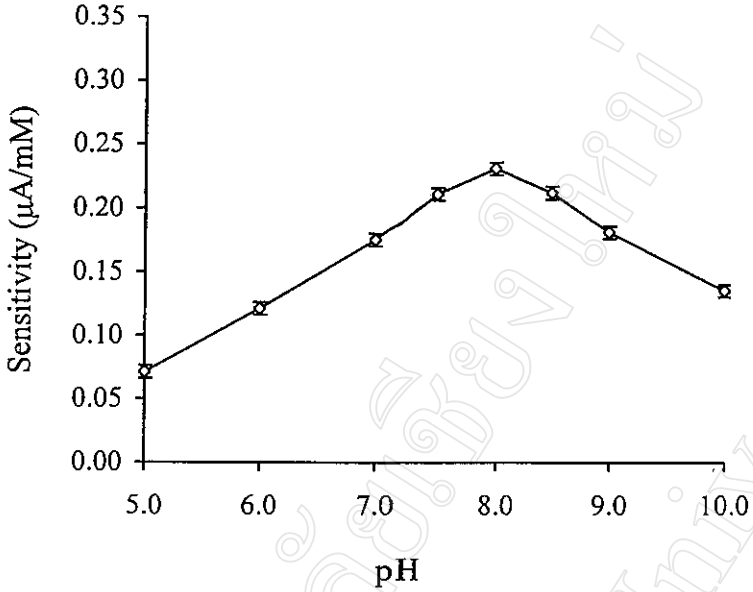

Figure 3.28 The influence of pH value on glycolic acid bioelectrode in BIA system [applied potential, 0.00 V (*vs.* Ag/AgCl); 30 μl of 0.1-1.0 mM glycolic acid in 0.05 M NaH_2PO_4-NaOH buffer; 16%(w/w) sunflower leaf tissue and 5%(w/w) ferrocene; stirring rate, 100 rpm; tip-electrode distance, 1 mm]

3.3.2.3 Effect of buffer system in BIA system

The effects of five buffer types, NaH_2PO_4-NaOH, Na_2HPO_4-NaH_2PO_4, boric-Na_2HPO_4, tartaric-NaOH and tris-HCl, on the responses of bioelectrode were investigated (Table 3.37 and Figure 3.29). It was found that the NaH_2PO_4-NaOH buffer (pH 8.0) was the most efficient working buffer giving the highest response among the five types of buffers studied. Hence, it was employed for the further studies.

Table 3.37 Effect of buffer systems on glycolic acid bioelectrode in BIA system

Buffer systems	Current (μA)* at [glycolic acid] (mM)				Sensitivity** (μA mM^{-1})
	0.1	0.3	0.5	1.0	
NaH_2PO_4-NaOH	0.036	0.075	0.131	0.220	0.231
NaH_2PO_4-Na_2HPO_4	0.024	0.062	0.103	0.148	0.164
Boric-Na_2HPO_4	0.020	0.064	0.090	0.132	0.147
Tataric-NaOH	0.019	0.059	0.086	0.171	0.173
Tris-HCl	0.006	0.031	0.059	0.070	0.081

* = mean of triplicate

** = The sensitivity is defined as the slope (ΔI/[glycolic acid]) of a plot of response *versus* glycolic acid concentration.

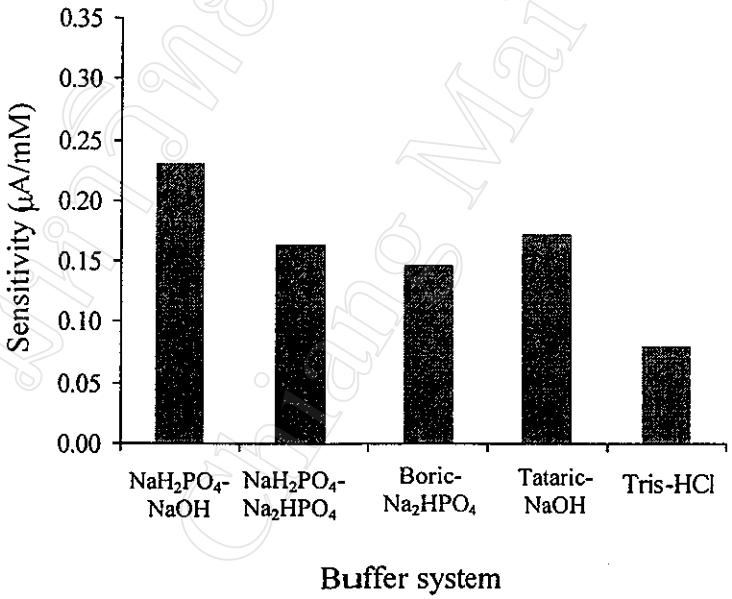

Figure 3.29 The dependent of buffer system on glycolic acid bioelectrode in BIA system [16%(w/w) sunflower leaf tissue and 5%(w/w) ferrocene; 30 μl of 0.1-1.0 mM glycolic acid; Each carrier buffer concentration was 0.05 M, pH 8.0; applied potential, 0.00 V (*vs.* Ag/AgCl); stirring rate, 100 rpm; tip-electrode distance, 1 mm]

3.3.2.4 Effect of tissue loading in BIA system

The tissue loading has a pronounced effect upon the bioelectrode response to glycolic acid. Increasing the tissue composition of the bioelectrode from 10 to 16%(w/w) resulted in an increase in the bioelectrode sensitivity (Table 3.38 and Figure 3.30), reflecting the increase in the biocatalytic activity. However, the response times as well as the noise level gradually increased with increase in tissue content. The sensitivity of the bioelectrode decreased when the tissue loading was increased above 16% (w/w). This behavior is ascribed to the lowering of the electrical conductivity due to the reduction in graphite loading [79]. The tissue loading of 16%(w/w) was selected for the next experiments.

Table 3.38 Effect of tissue loading on glycolic acid bioelectrode in BIA system

Tissue loading (%w/w)	Current (μA)* at [glycolic acid] (mM)				Sensitivity** (μA mM^{-1})
	0.1	0.3	0.5	1.0	
0	0.000	0.001	0.001	0.002	0.002
10	0.021	0.065	0.096	0.134	0.151
12	0.019	0.063	0.114	0.182	0.192
14	0.017	0.067	0.119	0.215	0.219
16	0.025	0.079	0.134	0.230	0.239
18	0.015	0.071	0.129	0.230	0.235
20	0.021	0.064	0.127	0.222	0.227
24	0.047	0.080	0.129	0.191	0.210

* = mean of triplicate

** = The sensitivity is defined as the slope (ΔI/[glycolic acid]) of a plot of response *versus* glycolic acid concentration.

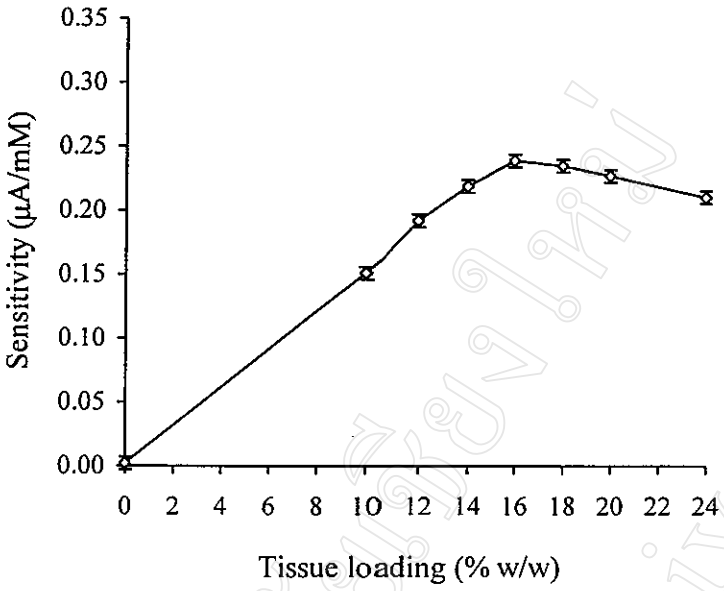

Figure 3.30 Effect of sunflower leaf tissue loading on glycolic acid bioelectrode in BIA system [5%(w/w) ferrocene mediator; 30 μl of 0.1-1.0 mM glycolic acid; 0.05 M NaH_2PO_4-NaOH buffer (pH 8.0); applied potential, 0.00 V (*vs.* Ag/AgCl); stirring rate, 100 rpm; tip-electrode distance, 1 mm]

3.3.2.5 Effect of mediator loading in BIA system

Table 3.39 and Figure 3.31 illustrates signals increasing substantially with increase in mediator loading from 2 to 5%(w/w). Further increase in mediator loading above 5%(w/w) resulted in a decrease in the bioelectrode sensitivity. Such a decrease when a larger amount of mediator is used in typical behavior of a mediator-based sensor and is ascribed to the lowering of the electrical conductivity. Moreover, the noise increased dramatically with an increase in mediator loading above 5%(w/w). Thus, a mediator loading of 5% was selected for further experiments.

Table 3.39 Effect of mediator loading on glycolic acid bioelectrode in BIA system

Mediator loading (%w/w)	Current (μA)* at [glycolic acid] (mM)				Sensitivity** (μA mM^{-1})
	0.1	0.3	0.5	1.0	
0	0.004	0.010	0.014	0.017	0.020
2	0.015	0.058	0.088	0.129	0.142
3	0.021	0.066	0.110	0.166	0.180
4	0.022	0.067	0.126	0.224	0.229
5	0.037	0.078	0.136	0.242	0.250
6	0.013	0.084	0.134	0.223	0.235
7	0.018	0.066	0.133	0.201	0.214
9	0.022	0.063	0.097	0.163	0.172

* = mean of triplicate

** = The sensitivity is defined as the slope (ΔI/[glycolic acid]) of a plot of response *versus* glycolic acid concentration.

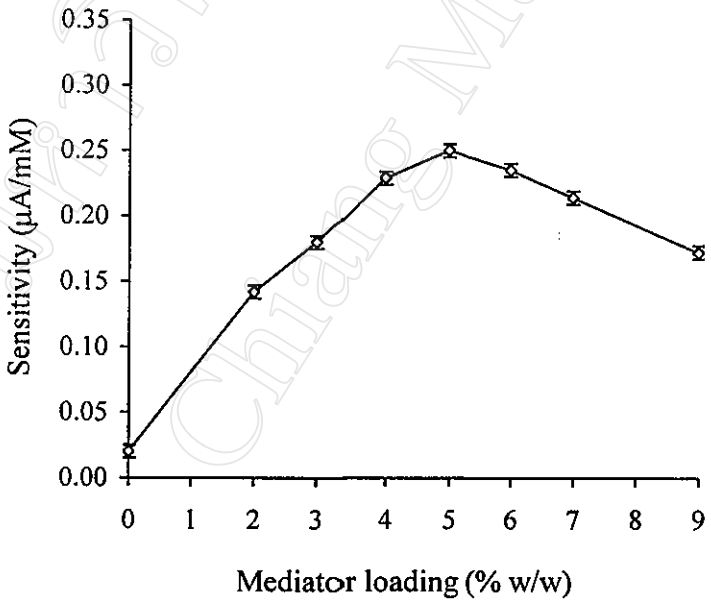

Figure 3.31 Effect of mediator loading on glycolic acid bioelectrode in BIA system [16%(w/w) sunflower leaf tissue; 30 μl of 0.1-1.0 mM glycolic acid; 0.05 M NaH_2PO_4-NaOH buffer (pH 8.0); applied potential, 0.00 V (*vs.* Ag/AgCl); stirring rate, 100 rpm; tip-electrode distance, 1 mm]

3.3.2.6 Effect of stirring rate in BIA system

The stirring rate dependence of the bioelectrode was pursued by varying the stirring rate from 50 to 300 rpm. Results were illustrated in Table 3.40 and Figure 3.32. It was found that the maximum sensitivity was obtained at the lowest stirring rate tested (50 rpm) then slightly decreased when the stirring rate increases up to 300 rpm. However, the maximum response time is obtained at flow rate of 50 rpm. The rapid decrease in bioelectrode response with the stirring rate is due to a reduced response time at the bioelectrode. Diffusion within the bioelectrode surface also plays an important role in the mass transfer because less substrate reaches the bioelectrode surface at the shorter response time. The stirring rate was therefore set at 75 rpm for compromise between bioelectrode sensitivity and response time.

Table 3.40 Effect of stirring rate on glycolic acid bioelectrode in BIA system

Stirring rate (rpm)	**Current (μA)* at [glycolic acid] (mM)**				**Sensitivity** (μA mM^{-1})**
	0.1	**0.3**	**0.5**	**1.0**	
50	0.049	0.163	0.214	0.305	0.345
75	0.048	0.130	0.193	0.293	0.321
100	0.052	0.116	0.160	0.237	0.264
150	0.047	0.106	0.142	0.207	0.233
200	0.008	0.046	0.102	0.155	0.164
300	0.027	0.056	0.093	0.141	0.153

* = mean of triplicate

** = The sensitivity is defined as the slope (ΔI/[glycolic acid]) of a plot of response *versus* glycolic acid concentration.

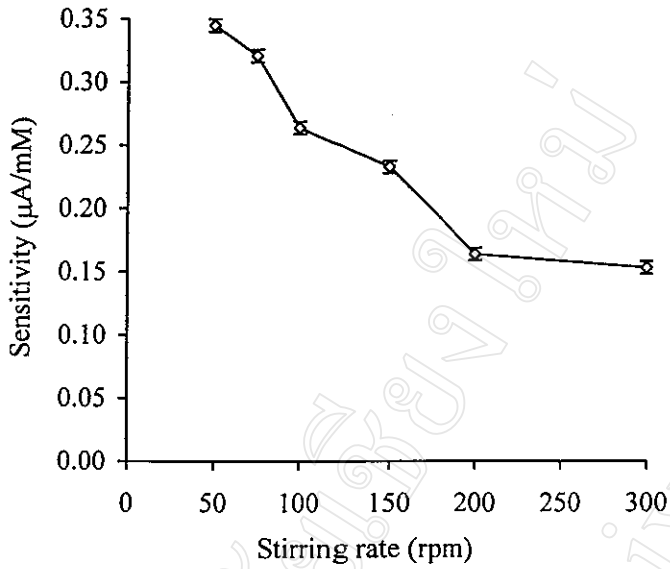

Figure 3.32 Effect of stirring rate on glycolic acid bioelectrode in BIA system [30 μl of 0.1-1.0 mM glycolic acid; 0.05 M NaH_2PO_4-NaOH buffer; applied potential, 0.00 V (*vs.* Ag/AgCl); 16%(w/w) sunflower leaf tissue and 5%(w/w) ferrocene; tip-electrode distance, 1 mm]

3.3.2.7 Effect of tip-electrode distance in BIA system

Table 3.41 and Figure 3.33 shows the dependence of the sensitivity upon the tip-electrode distance. It was found that the maximum sensitivity was obtained at the tip-electrode distance tested (1 mm) and decreased slightly as the tip moved further from the electrode.

Table 3.41 Effect of tip-electrode distance on glycolic acid bioelectrode in the BIA system

Tip-electrode distance (mm)	Current (μA)* at [glycolic acid] (mM)				Sensitivity** (μA mM^{-1})
	0.1	0.3	0.5	1.0	
1	0.039	0.107	0.173	0.304	0.316
2	0.027	0.088	0.153	0.249	0.262
3	0.015	0.074	0.105	0.190	0.197
4	0.011	0.043	0.078	0.147	0.148
5	0.012	0.040	0.074	0.128	0.132

* = mean of triplicate

** = The sensitivity is defined as the slope (ΔI/[glycolic acid]) of a plot of response *versus* glycolic acid concentration.

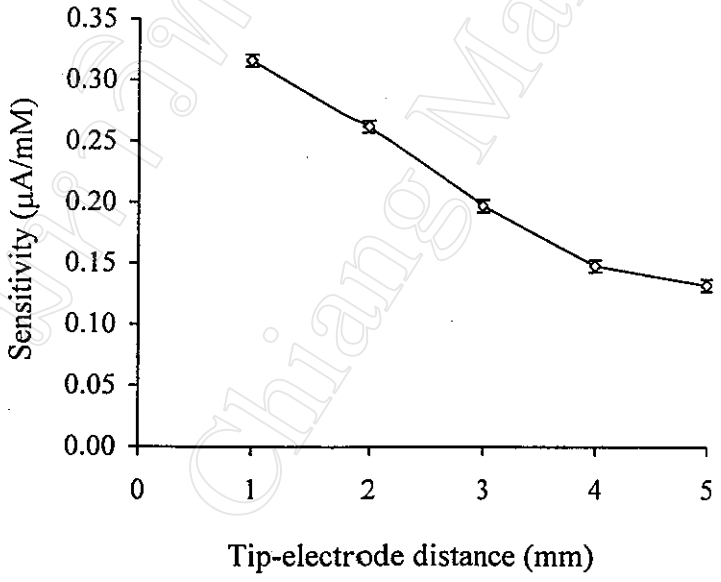

Figure 3.33 Effect of tip-electrode distance on glycolic acid bioelectrode in BIA system [30 μl of 0.1-1.0 mM glycolic acid ; 0.05 M NaH_2PO_4-NaOH buffer (pH 8.0); applied potential, 0.00 V (*vs.* Ag/AgCl); 16%(w/w) sunflower leaf tissue and 5%(w/w) ferrocene; stirring rate, 75 rpm]

3.3.2.8 Effect of injection volume in BIA system

The influent of the injection volume on the response of the bioelectrode was illustrated in Table 3.42 and Figure 3.34. It was found that the electrode response increased slightly when the sample volume was increased from 10 to 30 μl and then started to level off. Both similar profiles were previously reported for amperometric [56, 57], thermometric [62] and fluorometric [64] BIA detections. These behaviors are ascribed to the result of dispersion process (between the tip and the electrode) [57]. Based on the experimental results, the injection volume of 30 μl was selected.

Table 3.42 Effect of injection volume on glycolic acid bioelectrode in the BIA system

Injection volume (μl)	**Current (μA)* at [glycolic acid] (mM)**				**Sensitivity** (μA mM^{-1})**
	0.1	**0.3**	**0.5**	**1.0**	
10	0.025	0.108	0.170	0.262	0.283
20	0.031	0.110	0.186	0.285	0.307
30	0.037	0.122	0.192	0.302	0.324
40	0.031	0.133	0.199	0.301	0.328
50	0.037	0.104	0.199	0.308	0.328

* = mean of triplicate

** = The sensitivity is defined as the slope (ΔI/[glycolic acid]) of a plot of response *versus* glycolic acid concentration.

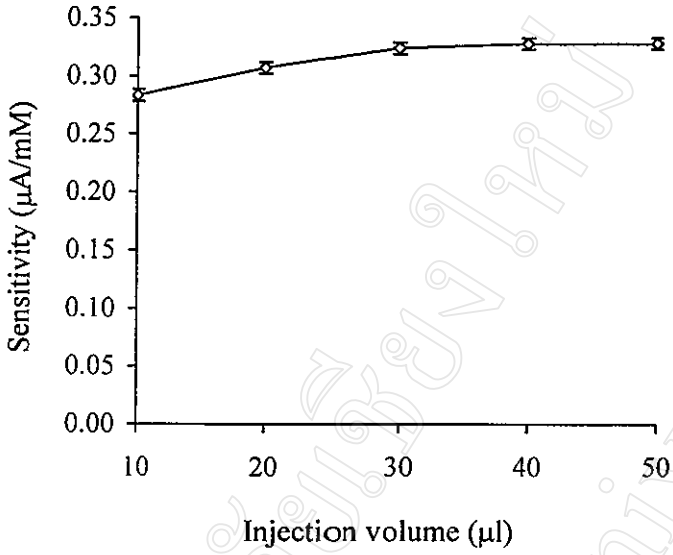

Figure 3.34 Effect of injection volume on glycolic acid bioelectrode in the BIA system [0.1-1.0 mM glycolic acid ; 0.05 M NaH_2PO_4-NaOH buffer (pH 8.0); applied potential, 0.00 V (*vs.* Ag/AgCl); 16%(w/w) sunflower) leaf tissue and 5%(w/w) ferrocene; stirring rate, 75 rpm; tip-electrode distance, 1 mm]

3.3.2.9 Summary of the optimum conditions in BIA system

The experimental variables of the sunflower based amperometric biosensor in batch injection system for glycolic acid determination had been optimized. The variables, their ranges studied and optimum conditions are shown in Table 3.43.

Table 3.43 Experimental variables, their ranges studied and optimum conditions for glycolic acid determination in BIA system

Experimental variables	**Range studied**	**Optimum conditions**
Applied potential (V *vs.* Ag/AgCl)	-0.15 to +0.15	0.0
pH	5.0 - 10.0	8.0
Buffer system	NaH_2PO_4-NaOH, Na_2HPO_4-NaH_2PO_4, boric-Na_2HPO_4, tartaric-NaOH and tris-HCl	NaH_2PO_4-NaOH
Tissue loading (%, w/w)	0 - 24	16
Ferrocene loading (%, w/w)	0 - 9	5
Stirring rate (rpm)	50 – 300	75
Tip-electrode distances (mm)	1 – 5	1
Injection volume (µl)	10 – 50	30

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3.3.3 Analytical characteristics of the proposed BIA method on the sunflower leaf tissue bioelectrode

3.3.3.1 Calibration, detection limit and reproducibility

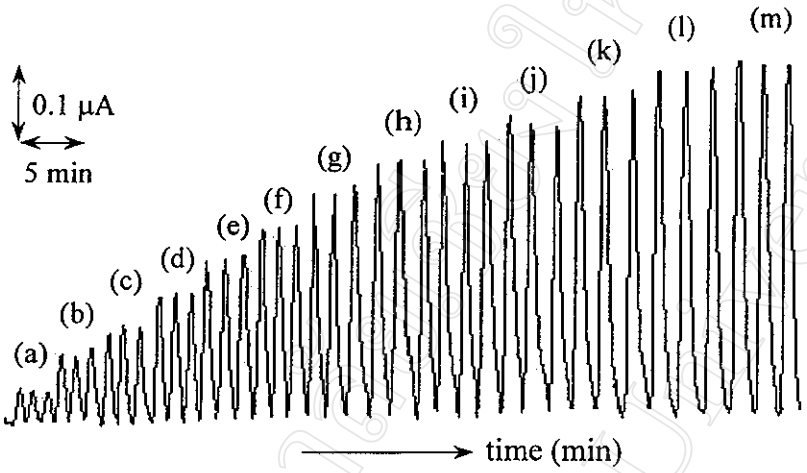

Figure 3.35 Typical response of glycolic acid in BIA system
[(a) 0.1, (b) 0.2, (c) 0.3, (d) 0.4, (e) 0.5, (f) 0.6, (g) 0.7 (h) 0.8, (I) 0.9, (j) 1.0, (k) 1.5, (l) 2.0, (m) 3.0 mM glycolic acid]

Figure 3.35 shows typical current response versus time signals of 0.1-3.0 mM glycolic acid obtained by using BIA under the optimum experimental conditions. It is seen that the bioelectrode exhibits an almost linearity of calibration up to 0.8 mM (Table 3.44 and Figure 3.36). The linear regression equation of the calibration graph is $y = 3.7\text{x}10^5\ x$ ($r = 0.997$). The sensitivity (slope of the initial linear range) corresponds to $3.7\text{x}10^5$ μA mM^{-1}. The signal-to-noise characteristics *(S/N)=3* indicates a detection limit of 0.01 mM glycolic acid. The method is highly reproducible with the relative standard deviation (R.S.D.) from the mean of 2.4 % obtained by replicate measurements (*n=15*) of 0.5 mM using the same bioelectrode (Table 3.45). Five-independently made bioelectrodes showed acceptable bioelectrode reproducibility with a R.S.D. of 4.3% (Table 3.46).

Table 3.44 Peak heights of standard glycolic acid in BIA system

Glycolic acid concentration (mM)	Peak height (μA)			Mean (μA)	SD
	1	2	3		
0.1	0.041	0.041	0.041	0.041	0.000
0.2	0.074	0.085	0.087	0.082	0.007
0.3	0.118	0.118	0.116	0.117	0.001
0.4	0.160	0.149	0.154	0.154	0.005
0.5	0.199	0.181	0.194	0.191	0.009
0.6	0.225	0.211	0.230	0.222	0.010
0.7	0.254	0.255	0.256	0.255	0.001
0.8	0.273	0.287	0.280	0.280	0.007
0.9	0.302	0.304	0.304	0.303	0.001
1.0	0.330	0.325	0.313	0.322	0.009
1.5	0.377	0.367	0.372	0.372	0.005
2.0	0.429	0.421	0.424	0.425	0.004
3.0	0.446	0.434	0.436	0.439	0.006

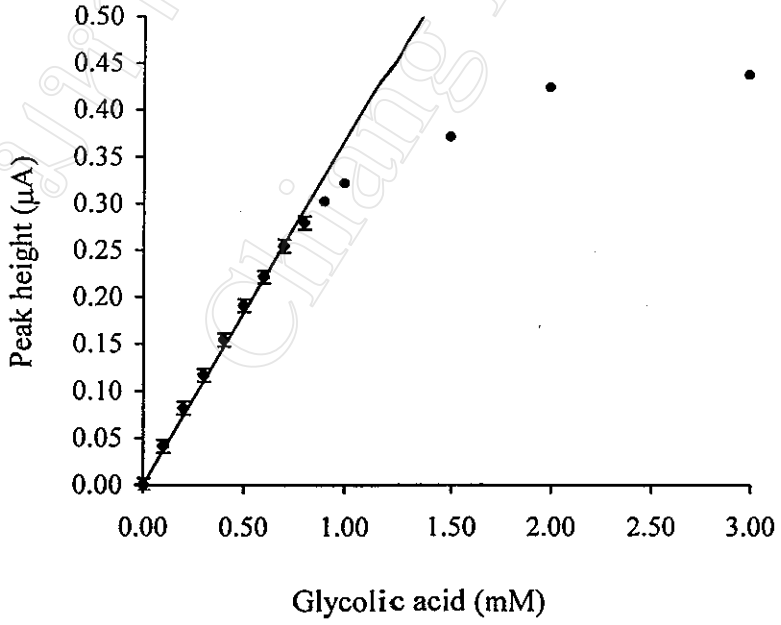

Figure 3.36 Calibration graph for glycolic acid in BIA system

Table 3.45 Replicate measurement of glycolic acid using the same bioelectrode in BIA system

Glycolic acid concentration (mM)	Current (μA)	Mean (μA)	SD	RSD (%)
1.0	0.175, 0.179, 0.173, 0.181, 0.173, 0.172, 0.177, 0.174, 0.182, 0.171, 0.173, 0.173, 0.170, 0.180, 0.170	0.175	0.004	2.306

Table 3.46 Replicate measurement of glycolic acid using five-independently made bioelectrode in BIA system

Glycolic acid concentration (mM)	Electrode No.	Current (μA)	Mean (μA)	SD	RSD (%)
1.0	1	0.179, 0.176, 0.175	0.169	0.007	4.3
	2	0.174, 0.175, 0.178			
	3	0.169, 0.166, 0.171			
	4	0.160, 0.170, 0.165			
	5	0.159, 0.162, 0.157			

3.3.3.2 Michaelis and Menten constant in BIA system

The apparent Michaelis-Menten constant K_m^{app}, and the maximum current density of glycolic acid sensor can be determined from the electrochemical Eadie-Hofstee form of the Michaelis-Menten equation. [67, 68]

$$I = I_{max} - K_m^{app}\left(\frac{I}{C}\right) \qquad (3.6)$$

Where I is the steady-state current, I_{max} is the maximum current measured under conditions of enzyme saturation and C is glycolic acid concentration. The current response of standard glycolic acid and current/[glycolic acid] are shown in Table 3.47. The plot is graphically illustrated in Figure 3.37. The K_m^{app} and I_{max} value for the bioelectrode were found to be 3.5 mM and 1.5 μA, respectively.

Table 3.47 Current response of standard glycolic acid and $\frac{\text{current}}{\text{[glycolic acid]}}$ in BIA system

Glycolic acid concentration (mM)	**Current (μA)***	$\frac{\textbf{Current}}{\textbf{[Glycolic acid]}}$, **(μA mM^{-1})**
.1	0.041	0.413
0.2	0.082	0.409
0.3	0.117	0.390
0.4	0.155	0.386
0.5	0.191	0.382
0.6	0.222	0.370
0.7	0.255	0.364
0.8	0.275	0.344

* = Mean of triplicate

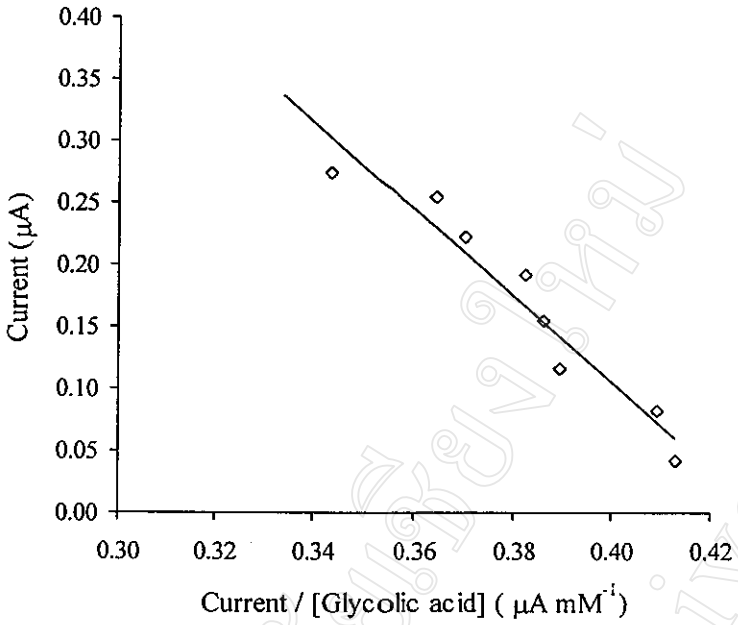

Figure 3.37 Eadie-Hofstee plot for the glycolic acid bioelectrode in BIA system

3.3.3.3 Response time of the bioelectrode in BIA system

Well-defined and sharp peaks, with rapid increase and decrease in the signals are observed. The half peak-width is 30 s. The short response time reflects the inherent advantage of the BIA technique over the batch measurements.

3.3.3.4 Stability of the bioelectrode in BIA system

In order to investigate the proposed electrode stability, aliquots of a standard solution of glycolic acid (0.5 mM) were injected continuously into the BIA system under the optimized conditions. Figure 3.38 shows the peak current of glycolic acid. It can be seen that the responses of the bioelectrode remain almost constant initially, up to 80 injections, because the enzyme is in excess. The bioelectrode responses decreased to 70% of the original value within 150 to 180 continuous injections. The loss of bioelectrode response is, therefore, ascribed to the leaching of ferrocene from the

bioelectrode and loss of the enzyme activity as described earlier in section 3.2.3.4.

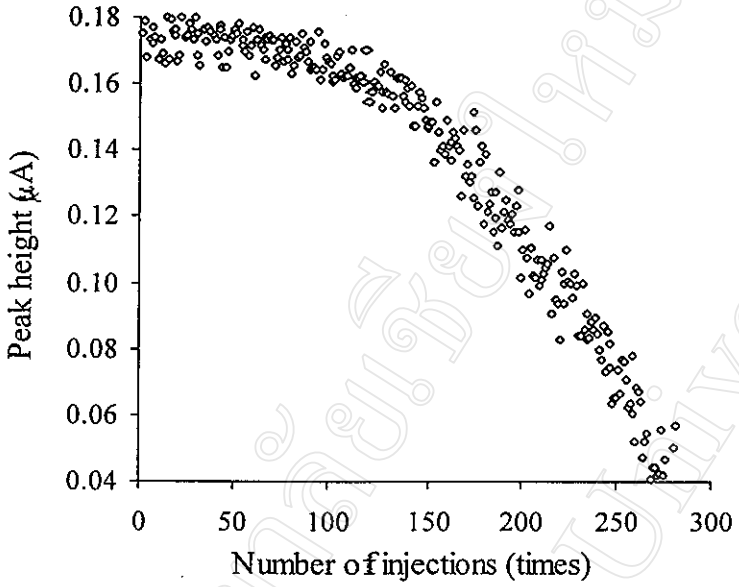

Figure 3.38 Stability of glycolic acid bioelectrode in BIA system

[30 μl, 0.5 mM glycolic acid; 0.05 M NaH_2PO_4-NaOH buffer (pH 8.0); applied potential, 0.00 V (*vs.* Ag/AgCl); stirring rate, 75 rpm; tip-electrode distance, 1 mm; 16%(w/w) sunflower leaf tissue and 5%(w/w) ferrocene]

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3.3.3.5 Interference studies in BIA system

A total of 14 possible interferences were used to evaluate the selectivity of the proposed bioelectrode incorporating with the BIA system. The current obtained for each potential interferent at 5 mM (except as otherwise stated) in the presence of glycolic acid at a concentration 0.5 mM was used as an indicator for bioelectrode selectivity in comparison with the glycolic acid reading alone. These results are listed in Table 3.48 which are slightly different from those obtained with the FI system (see section 3.2.3.5). It is evident that the bioelectrode is quite selective for glycolic acid as well as the FI system as described earlier. Of these substances studied, only ascorbic acid (0.1 mM) and uric acid, lactic acid (each 5 mM) interfered to a significant extent. As for most amperometric sensors, electroactive species such as ascorbic acid can easily be oxidized directly on the electrode surface at low potential, thus, changing the enzyme-mediated current. These effects clearly occur in the present work. However, in the presence of the lower concentration of ascorbic acid (0.005 mM) and lactic acid, uric acid (each 0.2 mM), it was found that no interference effects on the glycolic acid determination were observed. The small response to uric acid and lactic acid implies some cross-reactivity of enzyme glycolate oxidase towards uric acid and lactic acid [78, 84-85].

Table 3.48 Possible interferences tested with the glycolic acid bioelectrode in BIA system

Substances	Concentration (mM)	Current ratios [a,*]
Ascorbic acid	0.1	0.36
Tartaric acid	5.0	0.86
Salicylic acid	5.0	0.89
Oxalic acid	5.0	0.90
Glucose	5.0	0.92
Fructose	5.0	0.95
Succinic acid	5.0	0.96
Malonic acid	5.0	0.96
Maleic acid	5.0	0.99
Formic acid	5.0	1.11
Acetic acid	5.0	1.15
Citric acid	5.0	1.20
Glutamic acid	5.0	1.30
Uric acid	5.0	1.61
Lactic acid	5.0	1.71

[a] Current ratios for mixture of concentration of substance and 0.5 mM glycolic acid compared with 0.5 mM glycolic acid alone.
* = Mean of triplicate

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3.3.3.6 Percentage recoveries in BIA system

The accuracy of the bioelectrode response was evaluated by determining the recoveries of glycolic acid after standard addition of various concentrations of glycolic acid ranging from 0.1 to 0.4 mM to a 10-fold dilution of charcoal pretreated urine samples (as described in section 3.3.4). The bioelectrode shows satisfactory results with an average recovery of 97.4% (Table 3.49).

Table 3.49 Recoveries of glycolic acid added to a 10 fold-dilution of pretreated urine samples in BIA system

Sample No.	Glycolic acid concentration (mM)		%Recovery
	Added	Found*	
1	0.1	0.09	90.0
	0.2	0.19	95.0
	0.3	0.29	96.7
	0.4	0.39	97.5
2	0.1	0.10	100.0
	0.2	0.20	100.0
	0.3	0.30	100.0
	0.4	0.40	100.0
			Mean = 97.4

* = Mean of triplicate

3.3.4 Determination of glycolic acid in urine samples in BIA system

The BIA system with the proposed bioelectrode has been applied to the determination of glycolic acid in urine samples. The pretreatment step of the urine samples with charcoal is essential and recommended in order to eliminate any ascorbic acid and other electroactive compounds contained in the sample [86]. The 10 fold-dilution of urine samples and standard glycolic acid of 10.0 ml were mixed with 300 mg of activated charcoal and the charcoal was removed by centrifugation. The supernatant was then used for the glycolic acid assay by the proposed method (Table 3.44) and the results were compared with those obtained by HPLC method (appendix B) as mentioned earlier in section 3.2.4. A comparison of the results obtained is summarized in Table 3.50. A satisfactory agreement between the results was found with mean relative differences in the range 0.0 – 2.9%. Validation of the proposed method was also performed using t-test. There are 20-30 degree of freedom so the critical value of t-test is 2.09-2.04 at the confidence interval of 95%. The observed value of t-test is 0.08 (Table 3.52). It is show that the results obtained by the proposed method are in good agreement with those given by HPLC [87].

Table 3.50 Determination of glycolic acid in 10 fold-dilution of pretreated urine sample by the proposed method (BIA system)

Samples No.	Glycolic acid concentration (mM)	Mean (mM)	SD
1	0.179, 0.190, 0.191	0.186	0.007
2	0.346, 0.357, 0.355	0.353	0.006
3	0.350, 0.344, 0.356	0.350	0.006
4	0.413, 0.414, 0.429	0.419	0.009
5	0.477, 0.466, 0.484	0.476	0.009

Table 3.51 Determination of glycolic acid in 10 fold-dilution of pretreated urine sample by the proposed method (BIA system) and HPLC method [87]

Samples No.	Glycolic acid concentration (mM)		Relative difference (%)[c]
	Proposed method [a,*]	HPLC method [b,*]	
1	0.19	0.19	0.0
2	0.35	0.34	+2.8
3	0.35	0.35	0.0
4	0.42	0.42	0.0
5	0.48	0.47	+2.1

[c] = [(a – b) / a]x 100
* = Mean of triplicate

Table 3.52 Calculation for t-test (BIA system)

Methods	Glycolic acid concentration (mM)	Mean	SD	Degree of freedom	Observed value	Critical value
Proposed method	0.18, 0.19, 0.19, 0.35, 0.36, 0.36, 0.35, 0.34, 0.36, 0.41, 0.41, 0.43, 0.48, 0.47, 0.48	0.36	0.10	20-30	0.08	2.09-2.04
HPLC	0.19, 0.18, 0.19, 0.35, 0.33, 0.33, 0.37, 0.34, 0.32, 0.43, 0.42, 0.42, 0.48, 0.47, 0.47	0.35	0.10			