CHAPTER 3

RESULTS AND DISCUSSION

3.1 Electrochemical performance of the immobilization process

To investigate the modification steps of the electrode, cyclic voltammetric measurements were performed in permeable redox couple ($K_3[Fe(CN)_6]$ containing 0.1 M KCl) as shown in Figure 17. At the clean electrode surface, the redox couple was oxidized and reduced as shown in curve a. The redox peaks decreased when thioctic acid was self-assembled (curve b) and anti-*Salmonella* was immobilized (curve c) on the electrode. Finally, the pinholes on the electrode were blocked with 1-dodecanethiol as can be seen from the disappearance of the redox peaks in curve d.

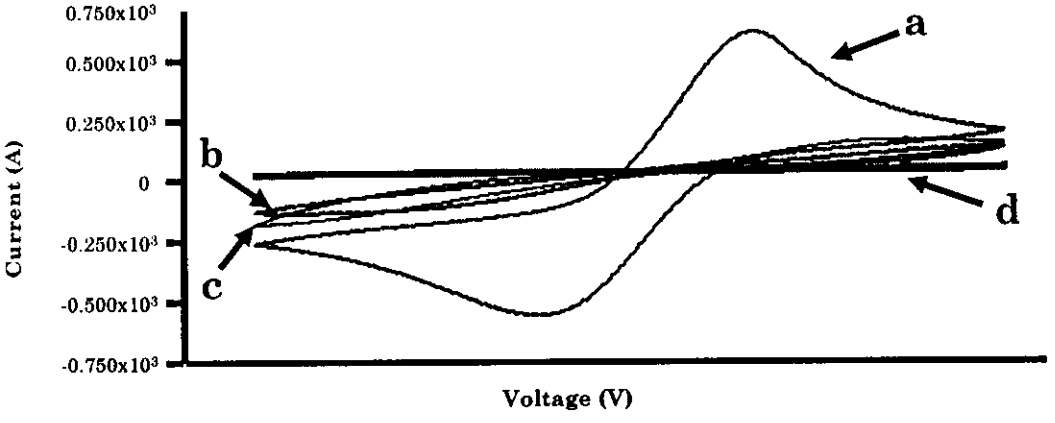


Figure 17 Cyclic voltammograms of a gold electrode obtained in 5 mM $K_3[Fe(CN)_6]$ containing 0.1 M KCl, (a) bare electrode, (b) thioctic acid covered gold, (c) anti-*Salmonella* modified thioctic acid couple gold, and (d) as in (c) but after 1-dodecanethiol treatment

3.2 Impedimetric measurement

Since impedance depends on frequency, the optimum frequency was investigated between 0.01 and 10,000 Hz with an alternative current voltage amplitude of 0.01 V. The optimum frequency was chosen from the region where the plot of impedance vs. log frequency is a straight line with a slope of about -1 and the phase angle closest to -90° as shown in Figure 18. The experiments were carried out in a flow injection system with 9 electrodes and the average was 108 $\pm$ 2 Hz. Then 108 Hz was used in the following experiments in flow system.

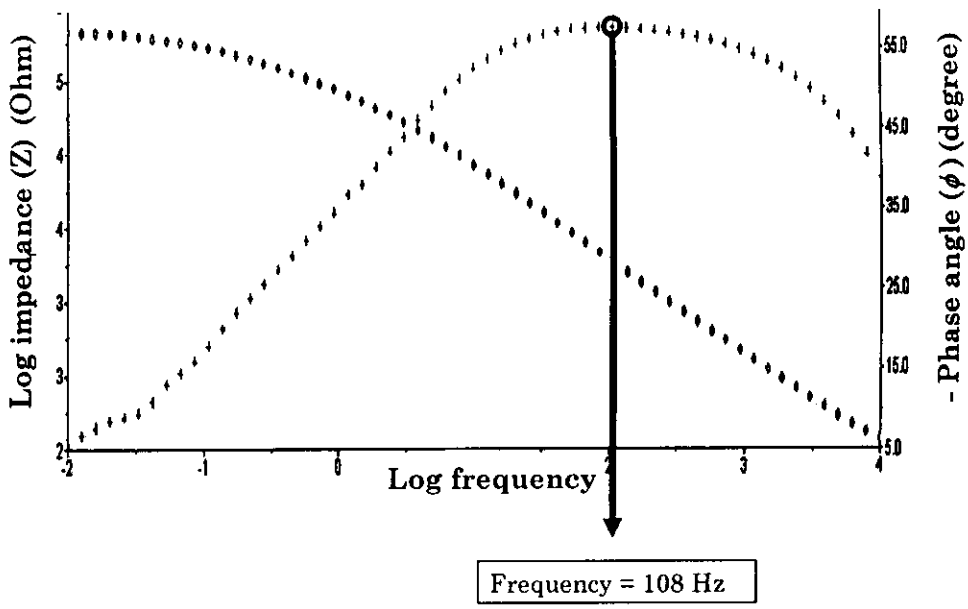


Figure 18 A bode plot obtained from an anti-*Salmonella* modified electrode in the flow system between log impedance (+) and phase angle (◊) with log frequency to determine the optimum frequency,

3.3 Optimization of operational conditions

Before investigating the impedimetric system a preliminary study to test the binding of the chosen species of *Salmonella* and antibody was done using the direct agglutination test. This technique is a general term for techniques which use the agglutination of particulate reagents as an indicator of the presence of an antigen-antibody reaction that was used to investigate their binding (Cappuccino and

Sherman, 2002). Twenty five microliters of 0.1 mg ml^{-1} anti-*Salmonella* was transferred with a micropipette into a 13 x 100 mm test tube and mixed with one milliliter of *Salmonella weltevreden*. The tube was incubated in a water bath at 48-50 °C for 18-24 h. The test showed a white precipitate which indicated the ability of the antibodies to bind with the chosen antigens.

To test the response of the modified electrode, standard solutions of *Salmonella* in the range of 1.00×10^2 to 1.50×10^7 cell/ml were injected into the biosensor system. The results are shown in Table 5 and Figure 19. Since the aim of this detection system is to obtain the lowest concentration of *Salmonella* and at 100 cell/ml the system could still provide a response, therefore, this was chosen as a concentration to optimize the operating conditions to obtain the lowest detection limit.

Table 5 Response of the modified electrode to various concentration of *Salmonella*.

Concentration of *Salmonella* (cell/ml)	**Impedance change (ΔZ") (Ohm)**
1.0×10^2	2.9 ± 0.2
3.4×10^2	4.5 ± 0.5
1.0×10^3	5.7 ± 0.6
3.1×10^3	7.0 ± 1.0
9.2×10^3	13.0 ± 1.0
2.8×10^4	16.0 ± 1.0
8.3×10^4	19.3 ± 0.6
2.5×10^5	24.7 ± 0.6
7.5×10^5	28.0 ± 1.0
1.8×10^6	32.0 ± 1.0
5.0×10^6	36.7 ± 0.6
1.5×10^7	38.0 ± 1.0
Sensitivity (Ohm/log cell/ml) of $1.0 \times 10^2 - 3.1 \times 10^3$ of *Salmonella*	1.24 ± 0.28
r of $1.0 \times 10^2 - 3.1 \times 10^3$ of *Salmonella*	0.996
Sensitivity (Ohm/log cell/ml) of $3.1 \times 10^3 - 5.0 \times 10^6$ of *Salmonella*	3.86 ± 0.15
r of $9.2 \times 10^3 - 5.0 \times 10^6$ of *Salmonella*	0.997

* 3 replications

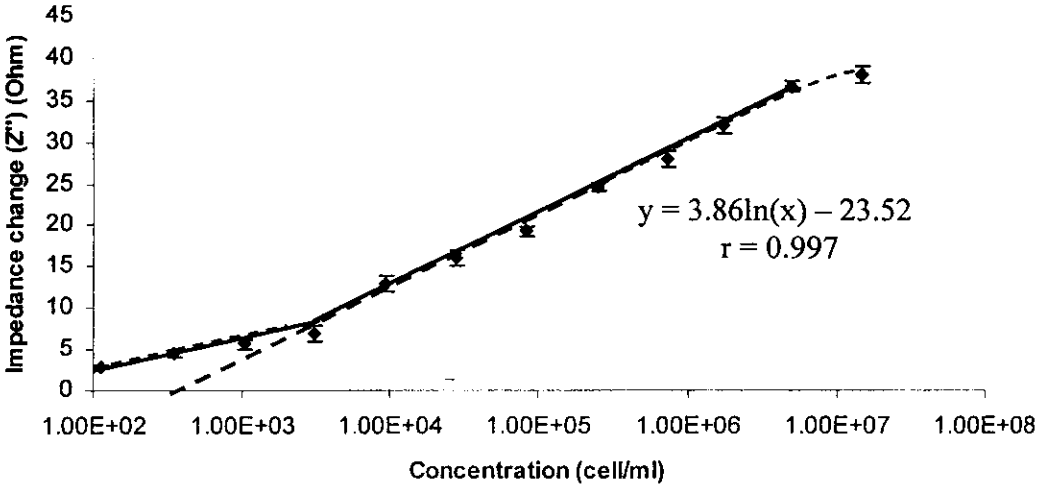


Figure 19 Response of the modified electrode to various concentration of *Salmonella*.

3.3.1 Type of regeneration solution

After the solution of *Salmonella* was injected, the cells bound to the antibodies and regeneration was needed to remove *Salmonella* from the antibodies so the electrode can be reused. The optimum regeneration solution was evaluated by considering the residual activity of the modified electrode. From the results in Table 6 and Figure 20, the regeneration solution in the group of high pH (NaOH) gave the highest % residual activity so NaOH was chosen for further investigation. This is probably because the antigen binds to antibody though weak hydrogen bonds (Subramanian *et al.*, 2005) and/or other non-covalent bonds i.e. electrostatic interactions, hydrophobic interactions and Van der Waals interactions (Byfield and Abuknesha, 1994) and these noncovalently bound molecules can be removed by NaOH (Kaplan *et al.*, 1997). NaOH was also used as the regeneration solution by other works such as Plomer *et al.* (1992) and Park *et al* (2000).

Table 6 Percentage residual activity using different types of regeneration solutions

Regeneration solution	% Residual activity
High ionic strength	
- 1 M NaCl	18 ± 1
- 1 M KCl	16 ± 1
- 1 M $MgCl_2$	15 ± 2
Low pH	
- Glycine/HCl pH 3.5	24 ± 1
- HCl pH 2.5	18 ± 1
- HCl pH 2.0	24 ± 6
High pH	
- 5 mM NaOH	43 ± 2
- 50 mM NaOH	34 ± 2

* 3 replications

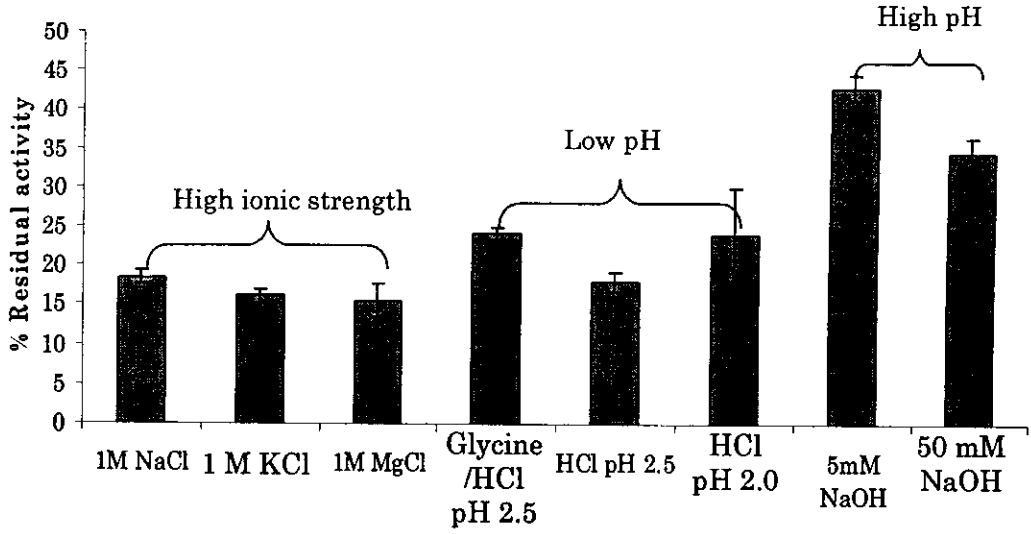


Figure 20 Percentage residual activity using different types of regeneration solutions

3.3.2 Concentration of regeneration solution

The optimum concentration of high pH regeneration solution was investigated. pH of NaOH corresponds to its concentration as shown in Table 7. The results (Table 7 and Figure 21) indicated that the % residual activity increased with NaOH concentration up to 20 mM and then decreased. This may cause by the denaturation of the antibody due to high pH solution (NaOH) (Blackburn, 1991). The optimum concentration of NaOH obtained from this experiment was 20 mM NaOH.

Table 7 Percentage residual activity at different concentrations of NaOH regeneration solution

Concentration of NaOH (mM)	**pH (approximate value)**	**% Residual activity**
5	11.7	43 ± 2
10	12.0	45 ± 1
15	12.2	82 ± 5
20	12.3	93 ± 5
25	12.4	39 ± 2
50	12.7	34 ± 2
100	13.0	34 ± 2
200	13.3	29 ± 2

- 3 replications

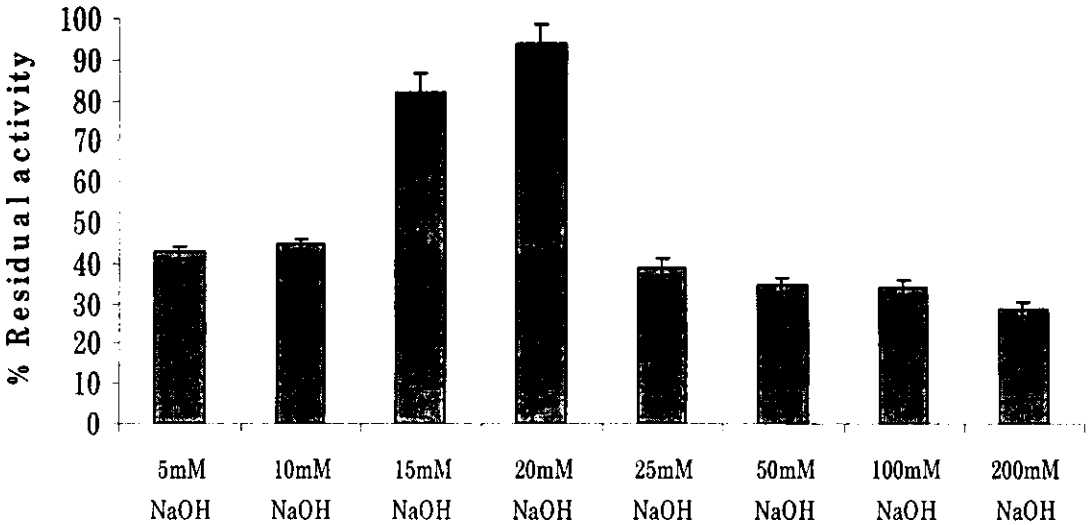


Figure 21 Percentage residual activity at different concentrations of NaOH regeneration solution

3.3.3 Sample volume

In the flow system, sample volume is also the main effect that needed to be investigated. From the results in Table 8 and Figure 22, 500 μl gave the highest response. However, the response at 400 and 450 μl were nearly the same as 500 μl and the analysis time of 400 μl was shorter by 2-3 min. Therefore 400 μl was chosen to be the optimum sample volume.

Table 8 Response of the biosensor system at different sample volume

Sample volume (µl)	Impedance change (ΔZ'') (Ohm)	Analysis time (min)
50	8.7 ± 1.5	8-10
100	14.0 ± 2.7	8-10
150	28.0 ± 3.6	10-12
200	43.0 ± 4.0	10-12
250	52.0 ± 3.0	11-12
300	64.0 ± 2.0	12-13
350	67.0 ± 1.0	15-18
400	68.7 ± 0.6	16-17
450	69.0 ± 1.0	16-18
500	69.3 ± 0.6	18-20

* 3 replications

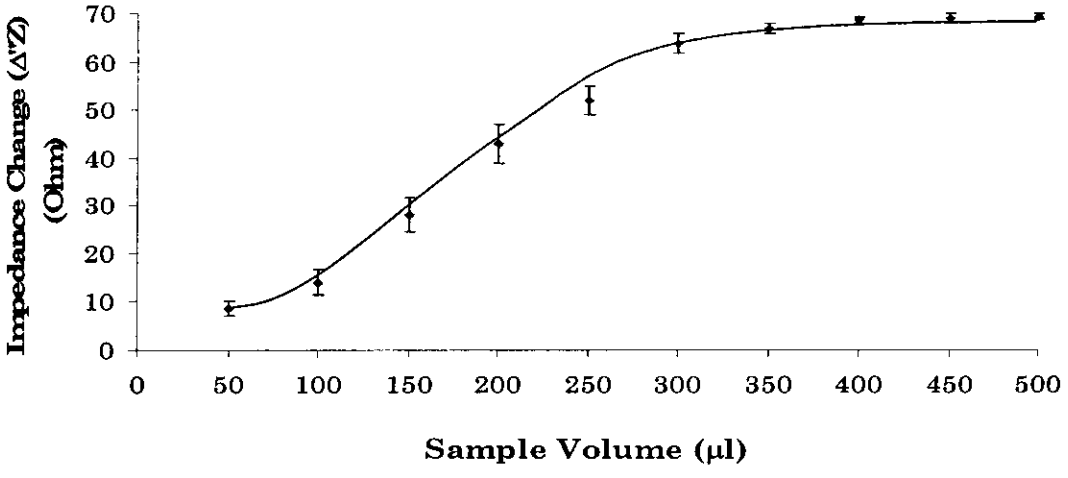


Figure 22 Response of the biosensor system at different sample volume

3.3.4 Flow rate

At lower flow rate the analyte had longer time to bind with antibodies on the modified electrode. Hence, the change of the impedance decreased when increasing the flow rate (Table 9 and Figure 23). The flow rate of 50 µl/min was chosen for further experiment although it took longer time it would help to improve the detection limit of *Salmonella* in the real sample. A flow rate lower than 50 µl/min was not investigated because of the limitation of the peristaltic pump.

Table 9 Response of the biosensor system at different flow rate

Flow rate (µl/min)	**Impedance change (ΔZ'') (Ohm)**	**Analysis time (min)**
50	148.0 ± 2.7	22-25
100	81.3 ± 5.5	20-22
150	53.0 ± 3.6	18-20
200	49.3 ± 1.2	16-17
250	39.0 ± 2.0	15-16
300	28.7 ± 2.5	10-12
350	19.2 ± 2.0	8-10
400	15.3 ± 3.5	7-8

* 3 replications

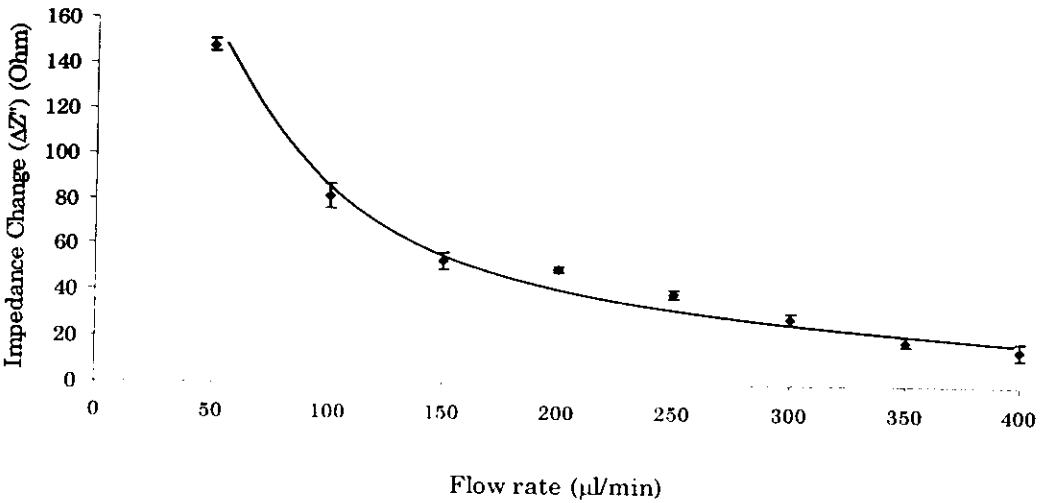


Figure 23 Response of the biosensor system at different flow rate

3.3.5 Type of buffer

Three types of buffer solution, *i.e.*, sodium phosphate buffer saline, potassium phosphate buffer saline and Tris-HCl, were investigated (Table 10 and Figure 24). Sodium phosphate buffer saline was chosen for further experiment because it gave the highest response and sensitivity. This is because the ionic strength of both sodium and potassium phosphate buffer saline are higher than Tris-HCl which relates to the amount of electron transfer (Skoog and West, 1996). While sodium and potassium phosphate buffer saline have the same ionic strength but the electrical resistivity of sodium solution is lower than that of potassium (Giancoli, D.C., 1995) therefore this might cause a better electron transfer in sodium phosphate buffer saline.

Table 10 Response of the biosensor system for different type of buffer solution

Concentration of *Salmonella* (cell/ml)	Impedance change (ΔZ") (Ohm)		
	Sodium phosphate buffer	Potassium phosphate buffer	Tris-HCl
1.0×10^2	26.7 ± 1.5	20.7 ± 0.9	5.1 ± 4.7
3.4×10^2	39.3 ± 2.2	31.8 ± 2.0	17.0 ± 3.5
1.0×10^3	51.0 ± 1.0	43.8 ± 1.0	30.0 ± 3.5
3.1×10^3	89.3 ± 6.5	74.9 ± 3.6	47.6 ± 2.4
Sensitivity (Ohm/log cell/ml)	17.5 ± 4.3	15.3 ± 3.0	12.3 ± 1.7
R	0.950	0.961	0.994

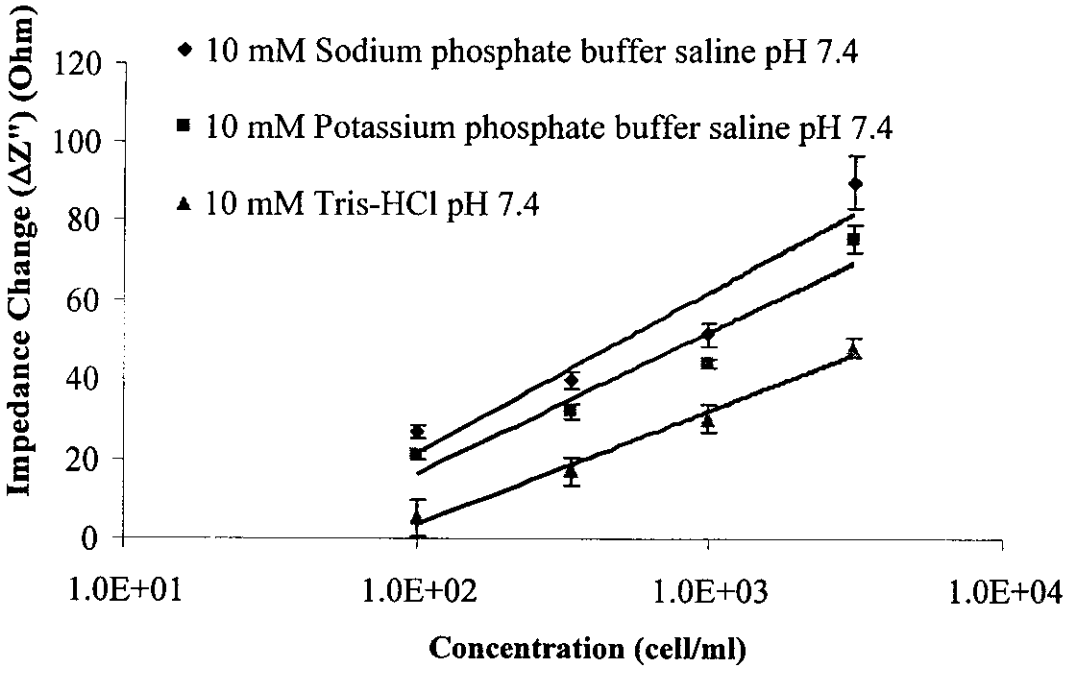


Figure 24 Response of the biosensor system for different type of buffer solution

3.3.6 pH of buffer

Many researches had used phosphate buffer at pH 7.4 for the impedimetric immunosensor (Ouerghi *et al.*, 2002, Su and Li, 2005, Susmel *et al.*, 2003). The effect of pH of phosphate buffer was investigated at pH 6.8 – 8.0. The results showed the highest response at pH 7.4 (Table 11 and Figure 25). This may be because one of the forces of binding this affinity pair is electrostatic, therefore, it depends on the charges on the antibody and antigen (Boehm *et al.*, 2000). The isoelectric point of *Salmonella* is 2.07 – 3.65 (Harden and Harris, 1953) and the isoelectric point of anti-*Salmonella* is 5.8 – 8.8 (Bradford *et al*, 1998; Govinden *et al*, 2006). Therefore, at pH 7.4 *Salmonella* had the negative charge and anti-*Salmonella* had positive charge and this might help with the binding. It is possible that at this pH the different charges on each side of he affinity binding pair enable maximum binding compare to other pHs.

Table 11 Response of the biosensor system at different pH of sodium phosphate buffer saline

pH of buffer	Impedance change (ΔZ") (Ohm)
6.8	13.3 ± 1.5
7.0	50.7 ± 8.0
7.2	96.3 ± 3.8
7.4	128.3 ± 4.0
7.6	78.0 ± 8.7
7.8	32.3 ± 3.8
8.0	13.0 ± 2.0

* 3 replications

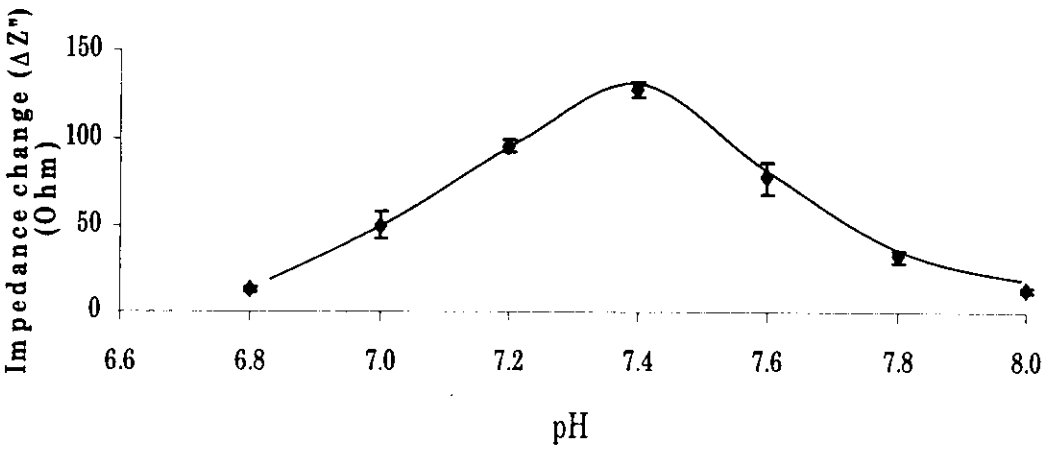


Figure 25 Response of the biosensor system at different pH of sodium phosphate buffer saline

3.3.7 Concentration of buffer

The ionic strength of buffer solution corresponds to the concentration of buffer (Bezerra *et al.*, 2003). When the concentration of buffer increases the ionic strength of buffer also increases which relates to the transfer of the electron. Table 12 and Figure 26 show that the change of impedance increased with the concentration of buffer. From the results, sodium phosphate buffer saline at the concentration of 100 to 400 mM gave nearly the same average impedance change and at the concentration of 100 mM gave the highest response. Then 100 mM phosphate buffer was chosen for further experiments.

Table 12 Response of the biosensor system at different concentrations of sodium phosphate buffer saline

Concentration of buffer (mM)	Impedance change (ΔZ'') (Ohm)
5	37.3 ± 2.1
10	41.7 ± 1.2
25	41.7 ± 0.6
50	41.3 ± 0.6
100	46.0 ± 1.0
200	45.7 ± 1.2
400	45.7 ± 0.6

* 3 replications

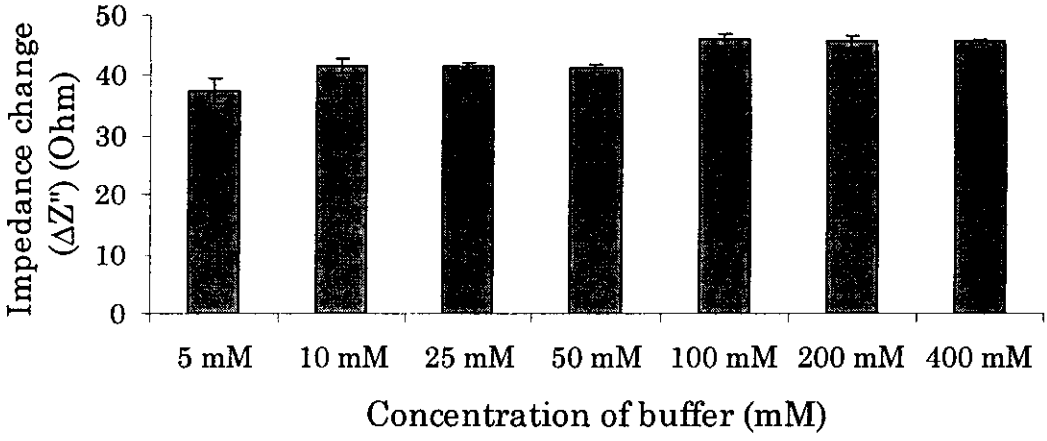


Figure 26 Response of the biosensor system at different concentrations of sodium phosphate buffer saline

The optimized parameters and values are summarized in Table 13.

Table 13 Optimum operational conditions

Parameters	Optimum
1. Type of regeneration solution	NaOH
2. Concentration of regeneration solution (mM)	20
3. Sample volume (μl)	400
4. Flow rate (μl/min)	50
5. Type of buffer	Sodium phosphate buffer saline
6. pH of buffer	7.4
7. Concentration of buffer (mM)	100

3.4 System

After the operational conditions were optimized, they were used to evaluate the performance of the system.

3.4.1 Linearity

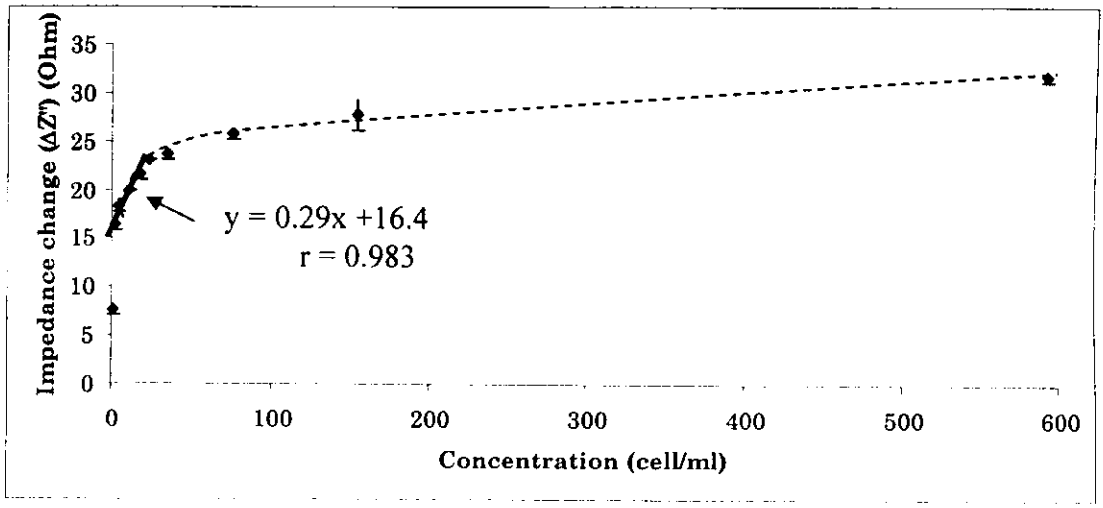
Standard solutions of *Salmonella* in the range of 1 cell/ml to 592 cell/ml were injected into the biosensor system under the optimum conditions to investigate the linearity. The plot of this investigation was done between the impedance change and concentration of *Salmonella* instead of the impedance change *vs.* the logarithm of *Salmonella* concentration in order to obtain more accurate result in the real sample. The linear range was between 2 and 24 cell/ml (Table 14 and Figure 27(b)) with a linear equation being impedance change (Ohm) $y = (0.29 \pm 0.04)$ Concentration (cell/ml) + (16.44 ± 0.30) (cell/ml). At higher concentrations the signal became constant (Figure 27(a)).

Table 14 Response of the biosensor system under the optimum conditions in the determination of linearity

Concentration of *Salmonella* (cell/ml)	**Impedance change (ΔZ") (Ohm)**
1	7.7 ± 0.6
2	16.3 ± 0.6
5	18.3 ± 0.6
11	20.0 ± 0
18	21.7 ± 0.6
24	23.0 ± 0
35	23.7 ± 0.6
76	25.7 ± 0.6
154	27.7 ± 1.5
592	31.7 ± 0.6
Sensitivity (Ohm/cell/ml) of *Salmonealla* 2 – 24 cell/ml	0.29 ± 0.04
R	0.983

* 3 replications

(a)



(b)

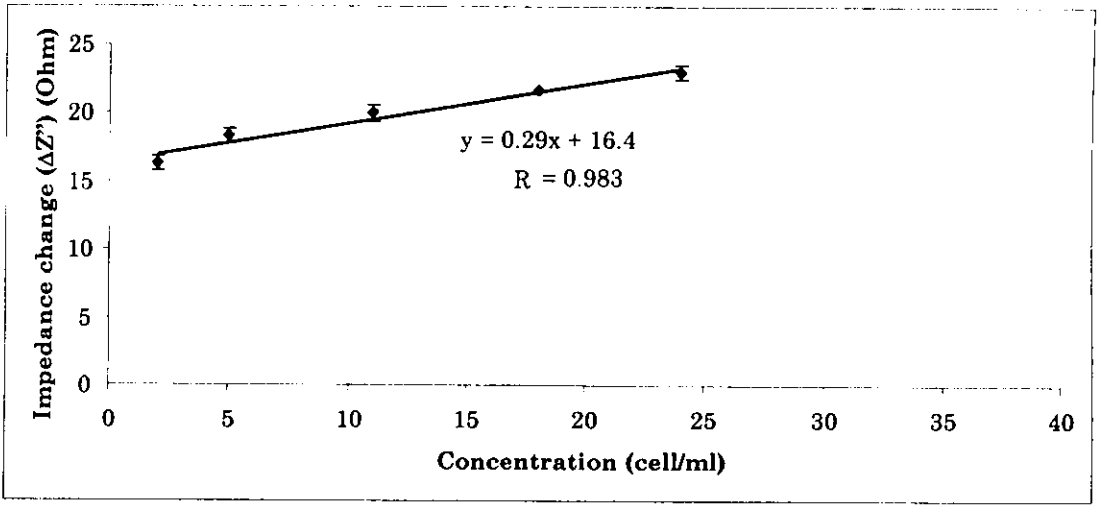


Figure 27 Response of the biosensor system under the optimum conditions in the determination of linearity

3.4.2 Limit of detection

To investigate the limit of detection, standard solutions of *Salmonella* at concentration between 4 and 20 cell/ml were injected into the biosensor system under the optimum conditions. The results are shown in Table 15 and Figure 28. The limit of detection (LOD) was calculated based on AOAC method (2004), that is

$$LOD = \frac{3 \times S_a}{b}$$

Where LOD = the limit of detection

S_a = the standard deviation of x blank results (X=10), which is 1.16

b = the slope of the calibration curve/ regression line, which is 1.49

So $LOD = \frac{3 \times 1.16}{1.49} = 2.35 \sim 2$ cell/ml

That is, the LOD of this system is 2 cell/ml.

Table 15 Response of the biosensor system under the optimum conditions

Concentration of *Salmonella* (cell/ml)	**Impedance change (ΔZ") (Ohm)**
4	10.0 ± 2.0
8	20.7 ± 0.6
15	28.7 ± 1.5
20	35.0 ± 1.0
Sensitivity (Ohm/log cell/ml)	1.49 ± 0.22
R	0.982

* 3 replications

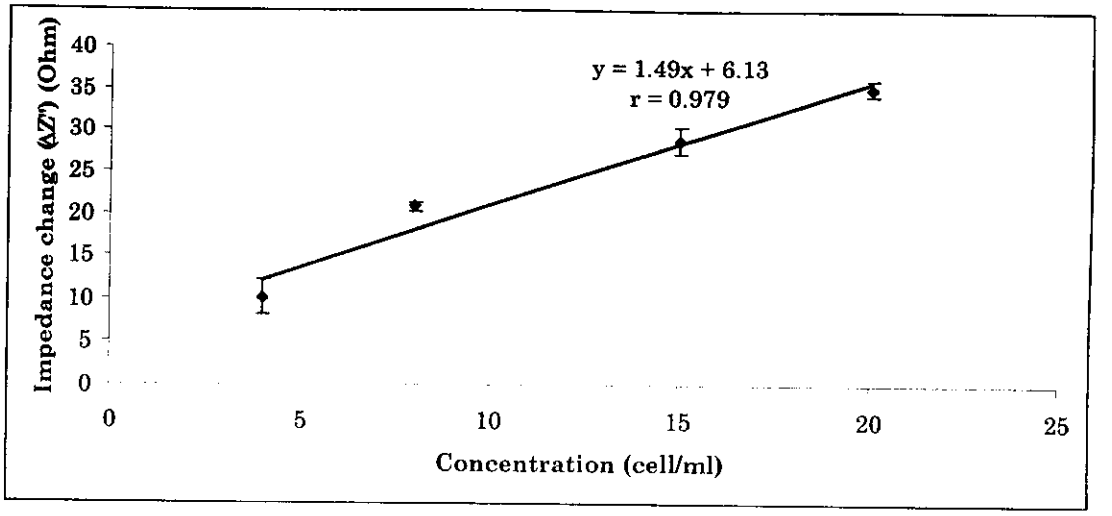


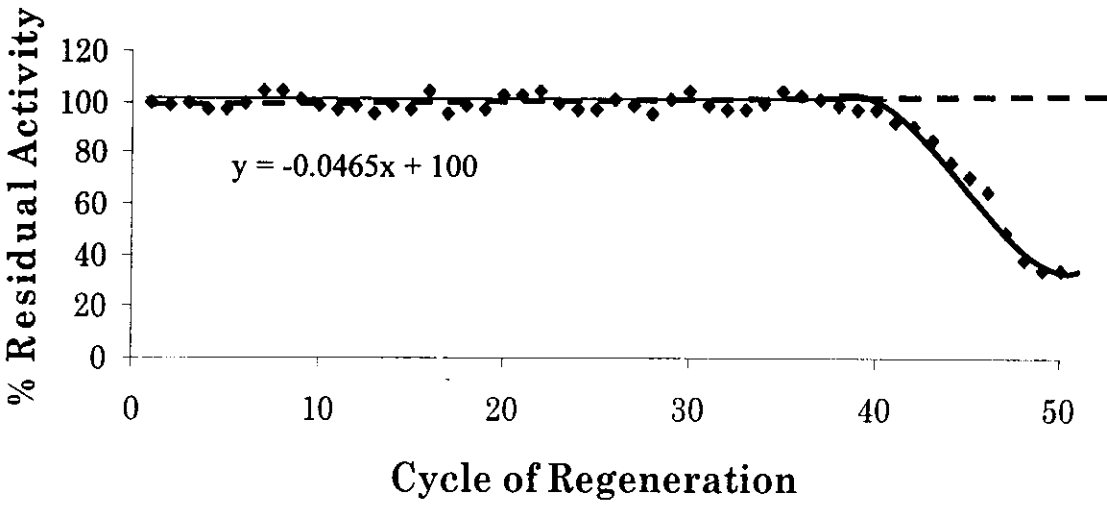
Figure 27 Response of the biosensor system under the optimum conditions

3.4.3 Stability of immobilized electrode

To test the stability of the modified electrode, 100 cell/ml of standard solution of *Salmonella* was injected into the biosensor system around 10 times a day for 5-6 days. The electrode was regenerated using the optimized regeneration solution to remove the analyte. The standard solution was then reinjected. Table 16 and Figure 29 show the % residual activity versus the cycles of regeneration. The electrode can be used up to 42 times for around 5 days with an average of % residual activity at 99.2 ± 3.2 . After that the % residual activity decreased rapidly. To prove that the % residual activity did not decrease because the SAM layer was destroyed, the electrode was tested by cyclic voltammogram measurement. Figure 30 shows the cyclic voltammograms before used (a) and after reused for more than 50 times (b). The signal of curve a and b were nearly the same. This indicates that the SAM layer was still on the gold electrode but after 42 times of reused the decrease of the % residual activity might cause by the lost of antibodies or their activity.

Table 16 Percentage residual activity of the modified electrode after regeneration

Number of injection	% Residual activity	Number of injection	% Residual activity
1	100	26	101
2	99	27	99
3	100	28	96
4	97	29	101
5	97	30	104
6	100	31	99
7	104	32	97
8	104	33	97
9	101	34	100
10	99	35	104
11	97	36	93
12	99	37	101
13	96	38	99
14	99	39	97
15	97	40	97
16	104	41	93
17	96	42	101
18	99	43	85
19	97	44	76
20	103	45	71
21	103	46	65
22	104	47	49
23	100	48	38
24	97	49	34
25	97	50	34



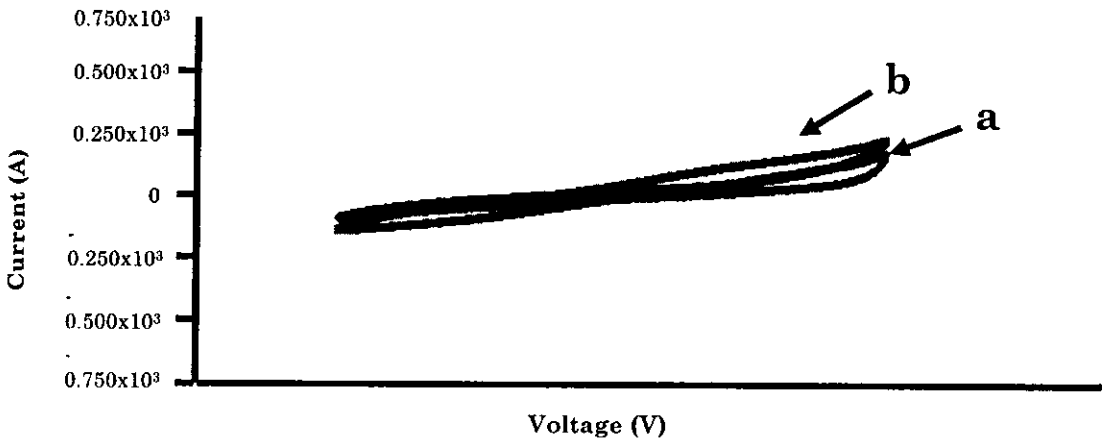


Figure 30 Cyclic voltammograms of a gold electrode obtained in a 5 mM $K_3[Fe(CN)_6]$ containing 0.1 M KCl, (a) the electrode was blocked by 1-dodecanethiol before use and (b) after reused for more than 50 times.

3.4.4 Selectivity

E.coli and *Shigella sonnei* which are the bacteria in the family *Enterobacteriaceae*, the same family as *Salmonella*, were used to investigate the selectivity of the system. Table 17 and Figure 31 show the response of *Salmonella*, *E.coli* and *Shigella sonnei* at the same concentrations. The impedance changes

obtained from *E.coli* and *Shigella sonnei* were much lower than those of *Salmonella*. The increase of the impedance change of *Salmonella* corresponded to the concentration. In contrast, the impedance change of *E.coli* and *Shigella* did not relate to the concentration and their responses were very low, the same as the blank. The results showed that the modified electrode was selective to target analyte.

Although in this work only one species of *Salmonella* was tested this system should be able to detect all types of *Salmonella*. This is because the antibody used in this work is a polyclonal antibody that can bind to all *Salmonella* serovars. Bokken *et* al. (2003) showed that this antibody can be used to detect all of the 53 serovars *Salmonella* being tested. That is, this antibody can detect any of the *Salmoenlla* serovars and is, therefore, suitable to be used for food regulation where *Salmonella* is not allowed to exist.

Table 17 Response of impedimetric biosensor to *Salmonella, E.coli* and *Shigella sonnei* and sodium phosphate buffer saline (blank)

Concentration (cell/ml)	**Impedance change (ΔZ") (Ohm)**
Salmonella	
2.0×10^0	15.3 ± 1.5
1.0×10^1	25.3 ± 2.5
1.2×10^2	33.3 ± 2.8
1.3×10^3	42.0 ± 2.0
Sensitivity (Ohm/[log cell/ml]) of *Salmonealla*	3.95 ± 0.56
Shigella sonnei	
1.0×10^0	4.3 ± 0.6
8.0×10^0	6.0 ± 1.0
1.1×10^2	6.8 ± 0.6
1.1×10^3	6.7 ± 1.2
E.coli	
3.0×10^0	6.0 ± 1.0
7.0×10^0	6.7 ± 2.1
1.3×10^2	6.7 ± 2.1
1.4×10^3	6.3 ± 1.2
Blank	4.0 ± 1.0
0×10^0	

* 3 replications

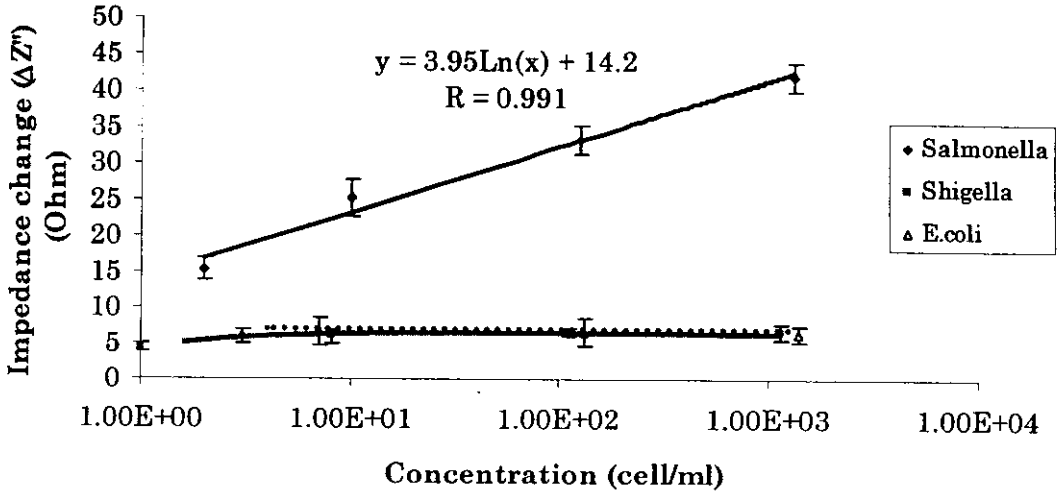


Figure 31 Response of impedimetric biosensor to *Salmonella, E.coli* and *Shigella sonnei* and sodium phosphate buffer saline (blank)

3.4.5 Effect of homogenized cell

Determination of *Salmonella* using the AOAC Official Method 967.26 requires living cells. In some instance, disrupted cell fragments of bacteria such as *E.coli* or *Salmonella* may also contain some toxin (Milner *et al.*, 1963) and this can not be detected with conventional method. However, it would be possible to detect with this system if the fragment contains the antigen that will bind to the antibodies on the electrode. Therefore, disrupted cells (homogenized cell) were also investigated and compared with unhomogenized cell. The results (Table 18 and Figure 32) showed that unhomogenized cells gave higher sensitivity than the homogenized cell. This might be because the size of disrupted cell is smaller than whole cell when it bound to the antibody it would give a lower response. That is, the biosensor system can be used to detect the homogenized cell although it gave lower response. The response to the mixture of disrupted cell and whole cell was lower than that of only the whole cell because the antibodies might bind to the small fragments of the disrupted cell instead of the whole cell giving a smaller impedance change. Therefore, biosensor system is an efficiency technique which can be used to detect both disrupted cell and whole cell of the antigen while the standard method can not detect the disrupted cell.

Table 18 Response of whole cell and homogenized cell

Concentration (cell/ml)	Whole cell	Homogenized cell	Mixed (2+2)*
2.0×10^0	21.3 ± 1.5	13.3 ± 1.5	19.0 ± 3.6
1.3×10^1	36.0 ± 1.0	21.0 ± 1.7	33.7 ± 5.1
1.4×10^2	42.7 ± 0.6	27.7 ± 2.1	36.7 ± 4.9
1.3×10^3	57.7 ± 1.5	33.0 ± 1.0	52.7 ± 4.5
Sensitivity (Ohm/[log cell/ml]	5.29 ± 0.64	3.00 ± 0.42	4.75 ± 1.28
R	0.986	0.993	0.966

* Mix (2+2) = 2 fold of homoginized cell + 2 fold of whole cell

** 3 replications

Table 19 Response of the mixed cell

Concentration (cell/ml)	Mixed (1+1)*
1.0×10^0	4.0 ± 3.6
6.5×10^1	13.3 ± 5.1
6.8×10^1	18.7 ± 4.9
6.4×10^2	24.7 ± 4.5
Sensitivity (Ohm/[log cell/ml]	3.07 ± 0.43
R	0.966

* Mix (1+1) = 1 fold of homoginized cell + 1 fold of whole cell

** 3 replications

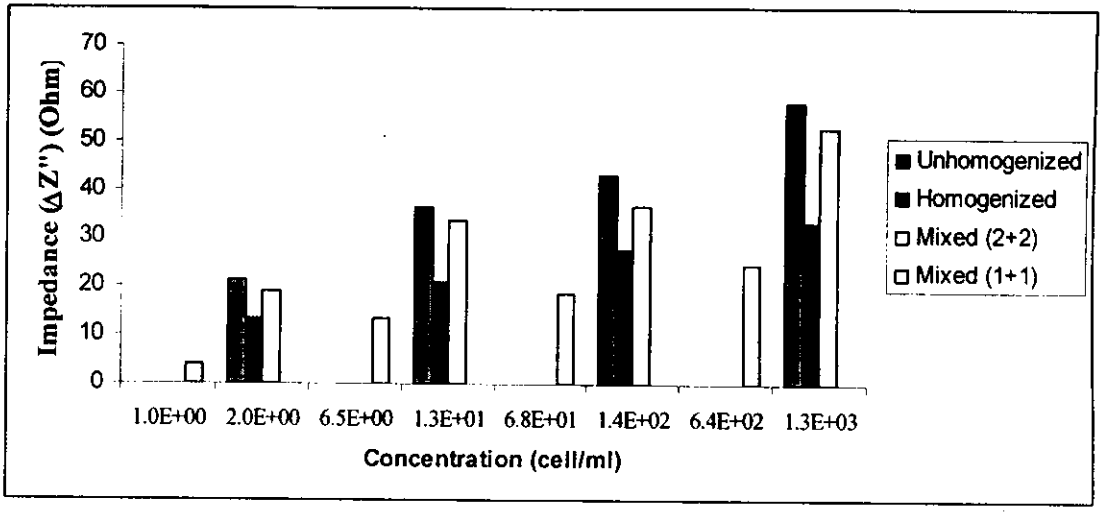


Figure 32 Response of whole cell and homogenized cell

3.5 Matrix interference

Matrix interference or matrix effect plays an important role in the accuracy and precision of a measurement. The sample matrix may lead to either a suppression or enhancement of the sample signal compared to the calibration signal for the same analyte quantity (Roper *et al.*, 2001). Matrix spiking was done by adding a known amount of *Salmonella* into the sample and then analyzed for the presence of spiked *Salmonella*. The slope of the standard and the spiked sample were compared for matrix interference and evaluated using statistic test. Various kinds of samples were tested for the matrix effect as shown in Tables 20-29 and Figures 33-39.

Table 20 Effect of matrix on the response of *Salmonella* in drinking water (Commercial 1and Commercial 2)

Concentration (cell/ml)	Response (Ohm)		
	Standard	Commercial 1	Commercial 2
4	10.0 ± 2.0	10.3 ± 1.5	11.7 ± 0.6
8	20.7 ± 0.6	19.7 ± 1.5	20.7 ± 1.5
15	28.7 ± 1.5	27.0 ± 1.0	28.7 ± 1.5
20	35.0 ± 1.0	35.7 ± 1.5	37.3 ± 0.6

* 3 replications

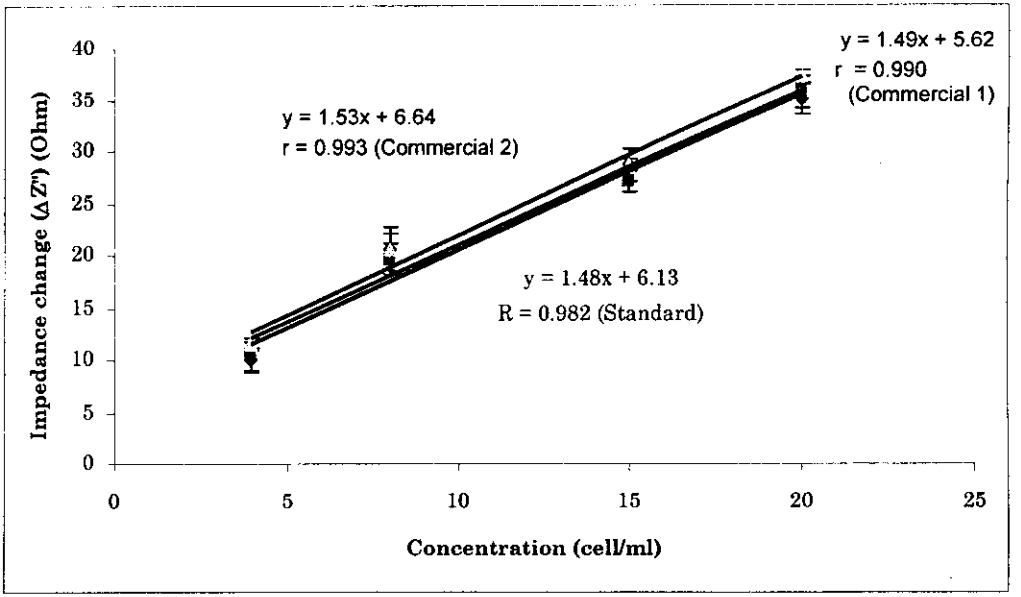


Figure 33 Effect of matrix on the response of *Salmonella* in drinking water (Commercial 1 and Commercial 2)

Table 21 Effect of matrix on the response of *Salmonella* in orange juice (Local 1)

Concentration (cell/ml)	Response (Ohm)	
	Standard	Sample
4	11.7 ± 0.6	56.7 ± 0.6
8	19.3 ± 0.6	64.7 ± 1.2
15	26.0 ± 1.0	71.0 ± 1.0
20	34.3 ± 0.6	80.3 ± 0.6

* 3 replications

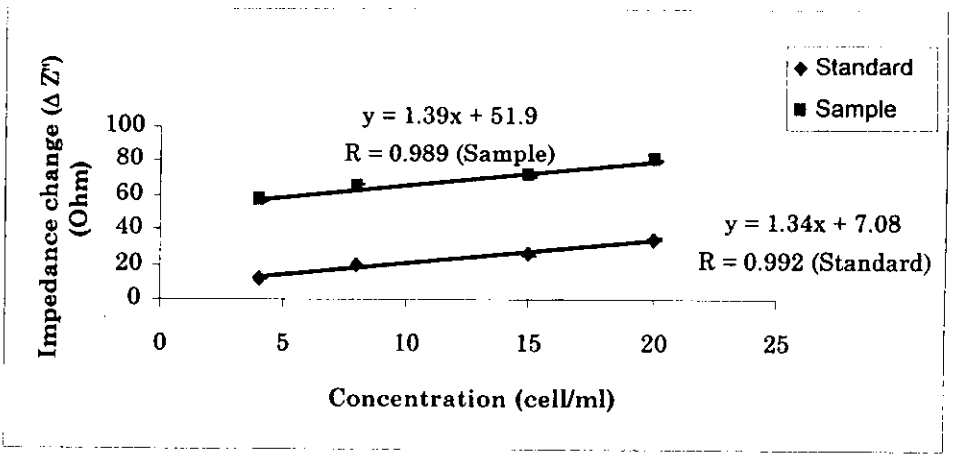


Figure 34 Effect of matrix on the response of *Salmonella* in orange juice (Local 1)

Table 22 Effect of matrix on the response of *Salmonella* in coconut juice (Local 2; 1st sampling)

Concentration (cell/ml)	Response (Ohm)	
	Standard	Sample
4	8.3 ± 0.6	29.7 ± 1.2
8	15.7 ± 0.6	36.3 ± 0.6
15	21.7 ± 0.0	43.7 ± 0.6
20	30.7 ± 0.6	51.3 ± 0.6

* 3 replications

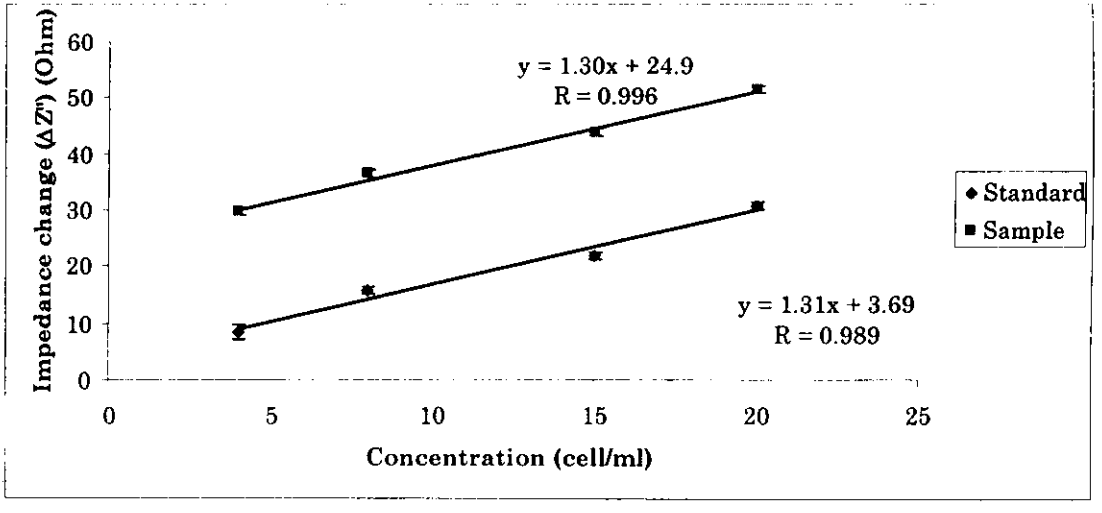


Figure 35 Effect of matrix on the response of *Salmonella* in coconut juice (Local 2; 1st sampling)

Table 23 Effect of matrix on the response of *Salmonella* in coconut juice (Local 2; 2nd sampling)

Concentration (cell/ml)	Response (Ohm)	
	Standard	Local 2
4	10.0 ± 1.0	43.7 ± 0.6
8	14.7 ± 0.6	50.7 ± 0.6
15	22.0 ± 1.0	56.7 ± 1.5
20	30.0 ± 1.0	64.0 ± 1.0

* 3 replications

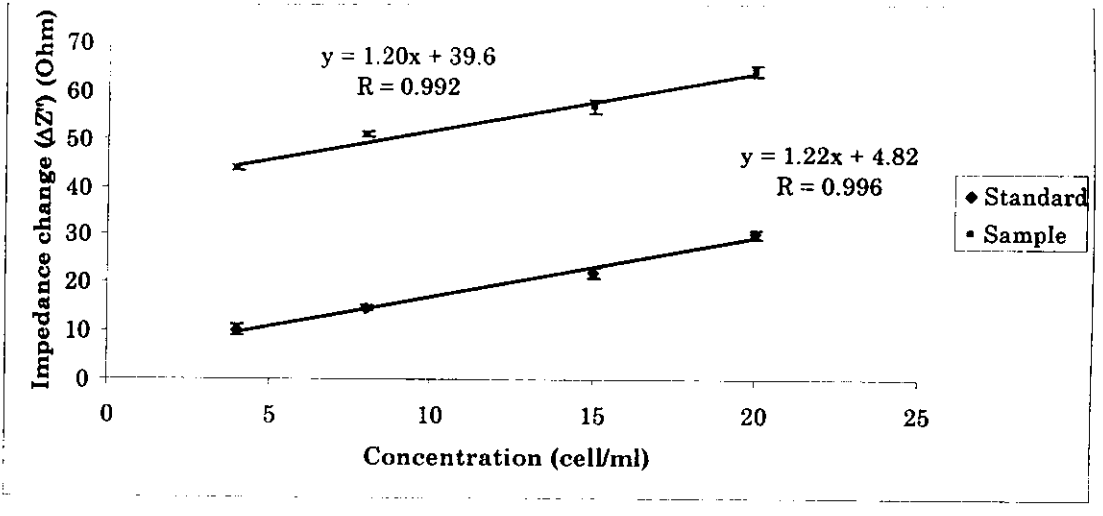


Figure 36 Effect of matrix on the response of *Salmonella* in coconut juice (Local 2; 2nd sampling)

Table 24 Effect of matrix on the response of *Salmonella* in coconut juice (Local 2; 3rd sampling)

Concentration (cell/ml)	Response (Ohm)	
	Standard	Local 2
4	8.3 ± 0.6	42.0 ± 1.0
8	14.7 ± 1.5	48.7 ± 1.5
15	25.0 ± 2.0	60.0 ± 2.0
20	32.0 ± 2.0	65.3 ± 1.5

* 3 replications

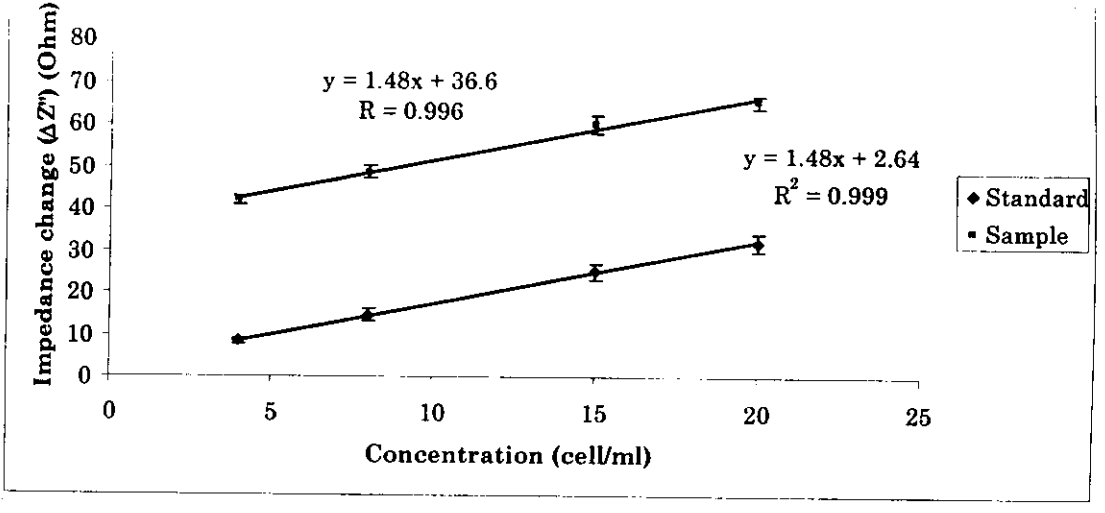


Figure 37 Effect of matrix on the response of *Salmonella* in coconut juice (Local 2; 3rd sampling)

Table 25 Effect of matrix on the response of *Salmonella* in coconut juice (Local 2; 4th sampling)

Concentration (cell/ml)	Response (Ohm)	
	Standard	Local 2
4	6.3 ± 0.6	41.7 ± 0.6
8	12.7 ± 1.5	47.0 ± 1.0
15	20.0 ± 1.0	55.3 ± 2.3
20	26.7 ± 1.5	64.0 ± 1.7

* 3 replications

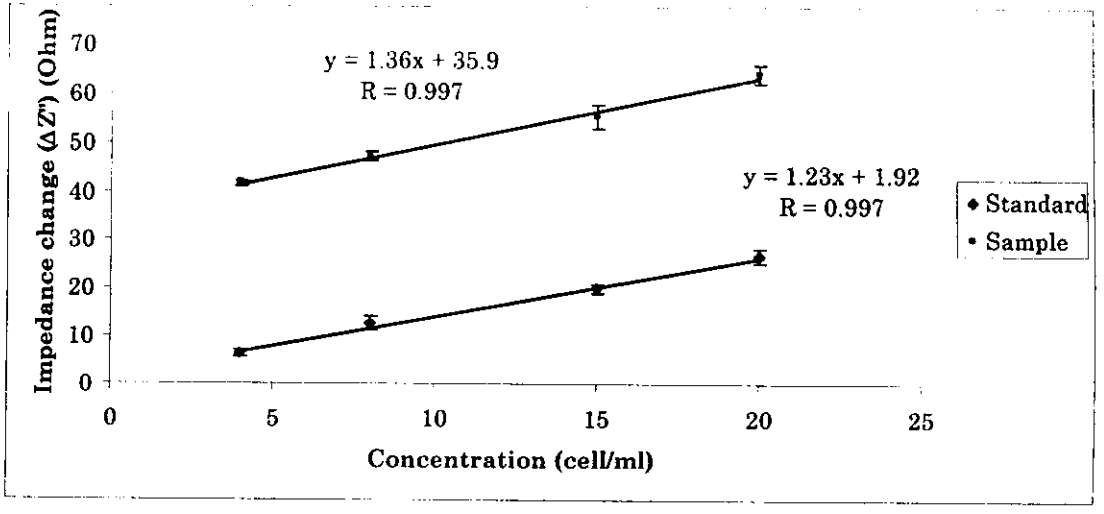


Figure 38 Effect of matrix on the response of *Salmonella* in coconut juice (Local 2; 4th sampling)

Table 26 Effect of matrix on the response of *Salmonella* in Orange juice (Commercial 3 and Commercial 4)

Concentration (cell/ml)	Response (Ohm)		
	Standard	Commercial 3	Commercial 4
4	12.0 ± 1.0	84.3 ± 1.5	82.7 ± 0.6
8	20.0 ± 1.0	93.7 ± 0.6	90.0 ± 1.0
15	30.0 ± 1.0	102.3 ± 0.6	99.0 ± 0.0
20	37.0 ± 0.0	110.3 ± 0.6	108.0 ± 1.0

* 3 replications

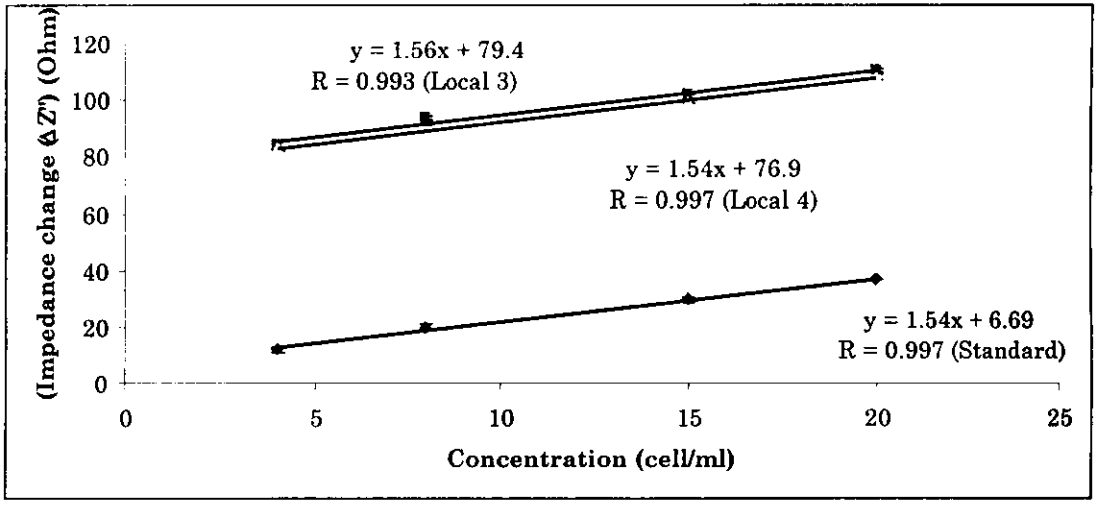


Figure 39 Effect of matrix on the response of Salmonella in Orange juice (Commercial 3 and Commercial 4)

Table 27 Effect of matrix on the response of *Salmonella* in Orange juice (Commercial 4; 1st sampling)

Concentration (cell/ml)	Response (Ohm)	
	Standard	Local 4
4	8.0 ± 1.0	84.7 ± 0.6
8	13.7 ± 0.6	87.7 ± 0.6
15	20.7 ± 2.1	96.0 ± 2.0
20	29.0 ± 1.0	104.0 ± 1.7

* 3 replications

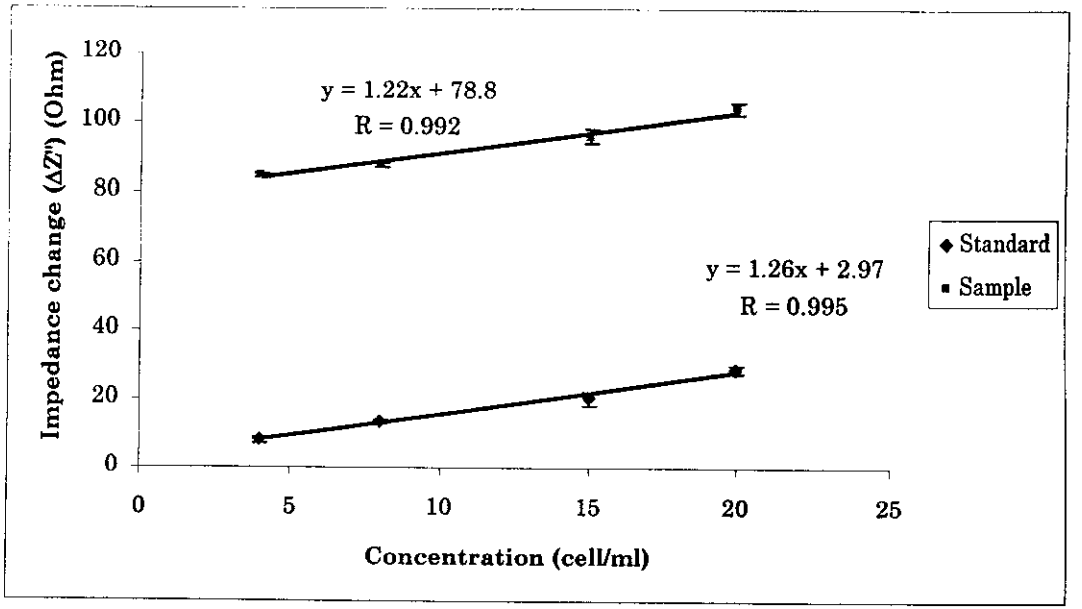


Figure 40 Effect of matrix on the response of *Salmonella* in Orange juice from (Commercial 4; 1st sampling)

Table 28 Effect of matrix on the response of *Salmonella* in Orange juice (Commercial 4; 2nd sampling)

Concentration (cell/ml)	Response (Ohm)	
	Standard	Local 4
4	6.0 ± 1.0	91.7 ± 0.6
8	12.7 ± 1.6	95.7 ± 1.2
15	18.3 ± 1.2	103.7 ± 1.5
20	24.3 ± 1.2	108.7 ± 2.1

* 3 replications

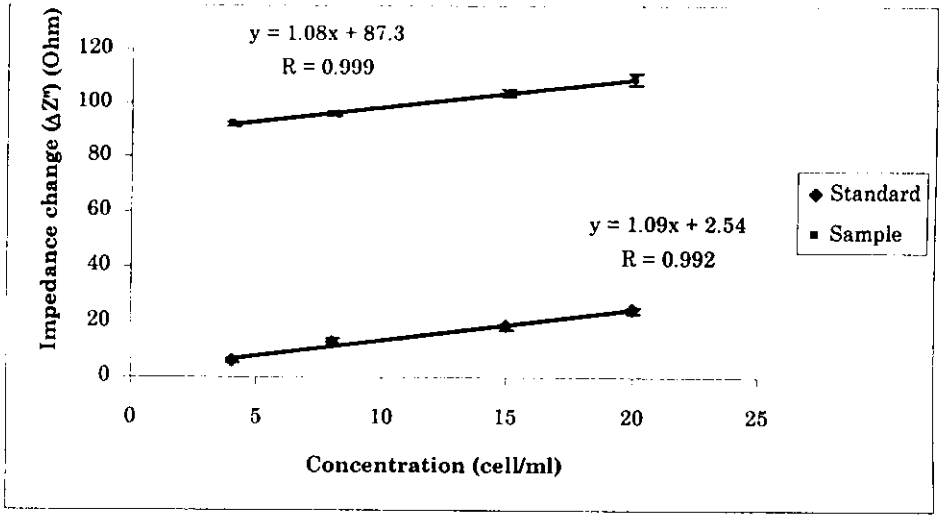


Figure 41 Effect of matrix on the response of *Salmonella* in Orange juice (Commercial 4; 2nd sampling)

Table 29 Effect of matrix on the response of *Salmonella* in Orange juice (Commercial 4; 3rd sampling)

Concentration (cell/ml)	Response (Ohm)	
	Standard	Local 4
4	8.3 ± 0.6	89.3 ± 0.6
8	17.0 ± 1.0	98.0 ± 1.0
15	25.3 ± 1.5	108.3 ± 2.1
20	35.0 ± 2.0	115.0 ± 2.0

* 3 replications

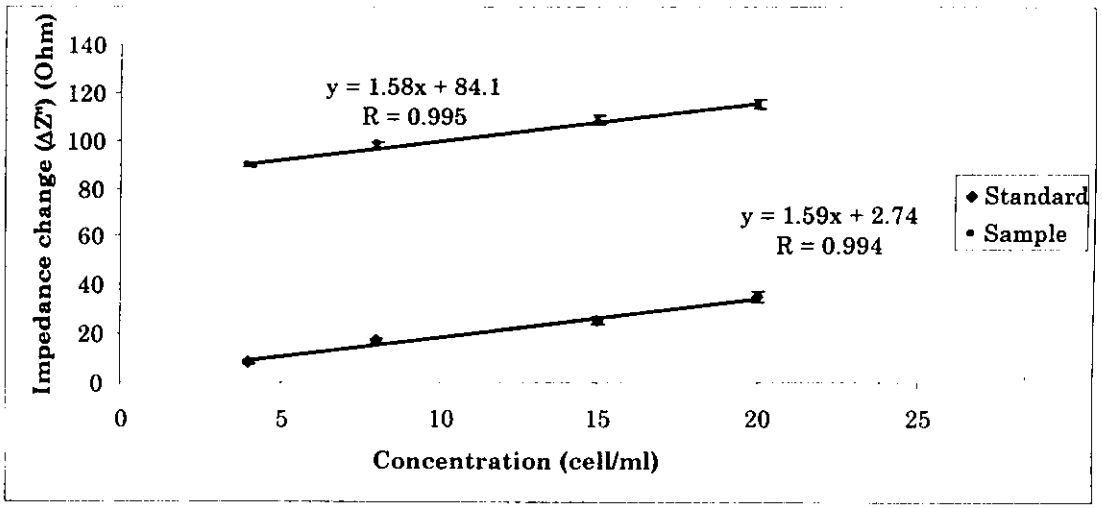


Figure 42 Effect of matrix on the response of *Salmonella* in Orange juice (Commercial 4; 3nd sampling)

The statistic ANOVA test (R software) indicates that the slope of regression line in each group of standard curve and matrix match calibration curve were not significantly different ($P < 0.05$). Therefore, there was not interference in the tested samples (Roper *et al.*, 2001). However, the results showed that the intercepts of the non-analyte samples were higher than those of the standard solutions indicated that the matrix gave a higher response. Then various types of sample were tested in the next experiment.

3.6 Determination of Salmonella in beverages

From the matrix effect study, various kinds of juice gave higher impedance change than blank so the response of different types of juice were studied. Before the samples were injected into the biosensor system, these samples were tested with the conventional method (AOAC method) to confirm that they did not contain *Salmonella*. Table 30 shows the response of various juices. The impedance changes were different for different types of sample. When the samples were of the same type, although from different sources, the impedance changes were nearly the same. Then the response of these uncontaminated samples was used to subtract from the measure signal to get the corrected response of the analyte. This corrected response was then used to determine the concentration of *Salmonella* from the standard solution calibration curve.

Table 30 Response of the modified electrode to various types of samples

Sample	Brand Name	% of Juice in the sample	pH	Impedance change ($\Delta Z''$) (Ohm)
Tamarind juice	Commercial 5	8	3.36	80.7 ± 0.6
Apple juice	Commercial 6	100	3.63	66.3 ± 2.1
Red grape juice	Commercial 7	100	3.64	62.7 ± 1.5
Orange juice	Commercial 3	10	3.10	80.3 ± 2.1
Orange juice	Commercial 4	100	3.36	81.7 ± 0.6
Orange juice	Commercial 8	10	3.28	82.3 ± 1.2
Orange juice	Commercial 9	Not indicated	3.26	82.0 ± 1.0
Orange juice	Commercial 10	36	3.18	80.0 ± 2.0
Coconut juice	Commercial 11	70	5.14	15.7 ± 0.6
Coconut juice	Commercial 12	100	5.17	14.7 ± 0.6

* 3 replications

3.6.1 Impedimetric biosensor system

Samples from various sources in Hat Yai, Songkhla were collected to determine the concentration of *Salmonella*. Before the samples were tested. *Salmonella* in carrying buffer (sodium phosphate buffer saline) in the range of 4 to 20 cell/ml were injected into the biosensor system under the optimum conditions. The calibration curve of the standard solution was plotted between the impedance change and concentration of *Salmonella*. Sample with no *Salmonella* (sample blank) and real sample were tested. The response of sample blank was then subtracted from the response of the real sample. The subtracted response was used to calculate for the concentration of *Salmonella* from the calibration curve. The results from the determination of real samples were shown in Table 31.

Table 31 Determination of *Salmonella* in sample using impedimetric biosensor system.

Sample	Amount of *Salmonella* (cell/ml)
Water (Commercial 1)	-0.6 ± 0.3
Water (Commercial 2)	0.2 ± 0.3
Orange juice (Local 1)	9.1 ± 0.4
Coconut juice (Local 2; 1st sampling)	38.3 ± 0.8
Coconut juice (Local 2; 2nd sampling)	6.3 ± 0.5
Coconut juice (Local 2; 3rd sampling)	7.4 ± 1.0
Coconut juice (Local 2; 4th sampling)	8.8 ± 1.7
Orange juice (Commercial 3)	-0.1 ± 0.3
Orange juice (Commercial 4; 1st sampling)	-0.1 ± 0.6
Orange juice (Commercial 4; 2nd sampling)	-2.4 ± 0.6
Orange juice (Commercial 4; 3rd sampling)	-2.3 ± 1.0
Orange juice (Commercial 4; 4th sampling)	-1.7 ± 1.3

* 3 replications

3.6.2 AOAC Official Method 967.26

The AOAC Official Method 967.26 was used to confirm the developed method. Table 31 shows the detection of *Salmonella* in samples using the two methods. The samples from Local 1 and Local 2 were found to be contaminated by *Salmonella*. But the results from the two techniques could not be statistically compared because the standard method can only indicate whether there is any *Salmonella* contaminated in the sample. In contrast, the biosensor system is an effective method that could be used to determine the amount of *Salmonella* in the samples. Though it still can not reach the detection limit at 1 CFU/ml but this developed technique gave lower detection limit when comparing to other techniques such as PCR technique developed by Chen *et al.* (1997) which detected *Salmonella* in sample at 3 CFU/25 ml, flow cytometry developed by McClelland and Pinder (1994) gave the detection limit at 10^3 CFU/ml and 350 CFU/ml for quartz crystal microbalance developed by Pathirana *et al.* (2000).

Table 32 The detection of *Salmonella* in the real samples using standard method compare with biosensor system

Sample	Source	Standard method	Biosensor system (cell/ml)
Water	Commercial 1	-	- 0.6 ± 0.4
Water	Commercial 2	-	- 0.2 ± 0.3
Orange juice	Commercial 3	-	- 0.1 ± 0.3
Orange juice	Commercial 4 1st sampling	-	- 0.1 ± 0.6
Orange juice	Commercial 4 2nd sampling	-	- 2.4 ± 0.6
Orange juice	Commercial 4 3rd sampling	-	- 2.3 ± 1.0
Orange juice	Commercial 4 4th sampling	-	- 1.7 ± 1.3
Tamarind juice	Commercial 5	-	-
Apple juice	Commercial 6	-	-
Red grape juice	Commercial 7	-	-
Orange juice	Commercial 8	-	-
Orange juice	Commercial 9	-	-
Orange juice	Commercial 10	-	-
Coconut juice	Commercial 11	-	-
Cocounut juice	Commercial 12	-	-
Orange juice	Commercial 13	-	-
Orange juice	Local 1	+	9.1 ± 0.4
Coconut juice	Local 2 1st sampling	+	6.3 ± 0.5
Coconut juice	Local 2 2nd sampling	+	7.4 ± 1.0
Coconut juice	Local 2 3rd sampling	+	8.8 ± 1.7
Coconut juice	Local 2 4th sampling	+	38.3 ± 0.8
Orange juice	Local 3	-	-
Orange juice	Local 4	-	-
Orange juice	Local 5	-	-
Coconut juice	Local 6	-	-
Coconut juice	Local 7	-	-

* 3 replications

3.7 Recovery

To determine percentage recovery, known amount of *Samonella* standard solution (4, 8, 15 and 20 cell/ml) were also spiked into samples collected from various sources in Hat Yai, Songkhla. The response of sample blank is similar to those in Table 30 and was used to subtract from the response of the sample. The concentration of the spiked sample was calculated from the calibration curve. The percentage of recovery was evaluated using following equation (Eurochem, 1998);

$$\%R = \frac{(CF - CU)}{CA} \times 100$$

Where CF = the concentration of analyte measured in the spiked sample

CU = the concentration of analyte measued in the blank

CA = the concentration of anaylte spike in the sample

The % recovery of the spiked in samples are shown in Tables 33 – 44. Table 45 summarized the % recovery in spiked sample. The percentage recoveries are in the range of 79 ± 11 to 129 ± 11 which are better than other work, for example, Lucore *et al.*, 2000 applied nucleic acid amplification method to detect *Listeria monocytogenes* and *Salmonella enteritidis* by concentrating the organisms from the food matrix before detection. The % recovery was found to be around 50%.

Table 33 Percentage recovery of *Salmonella* spiked in Water (Commercial 1) using impedimetric biosensor system.

Concentration (cell/ml)		% Recovery
Added	Found	
4	4.2 ± 0.7	104 ± 23
8	9.8 ± 0.7	122 ± 11
15	14.1 ± 0.5	94 ± 4
20	19.3 ± 0.7	97 ± 5

* 3 replications

Table 34 Percentage recovery of *Salmonella* spiked in Water (Commercial 2) using impedimetric biosensor system.

Concentration (cell/ml)		% Recovery
Added	Found	
4	4.2 ± 0.7	124 ± 9
8	9.8 ± 0.7	129 ± 11
15	14.1 ± 0.5	101 ± 6
20	19.3 ± 0.7	102 ± 2

* 3 replications

Table 35 Percentage recovery of *Salmonella* spiked in orange juice (Local 1) using impedimetric biosensor system.

Concentration (cell/ml)		% Recovery
Added	Found	
4	3.2 ± 0.3	79 ± 11
8	9.1 ± 0.7	114 ± 11
15	13.9 ± 0.6	92 ± 5
20	20.8 ± 0.3	104 ± 2

* 3 replications

Table 36 Percentage recovery of *Salmonella* spiked in coconut juice (Local 2; 1st sampling) using impedimetric biosensor system.

Concentration (cell/ml)		% Recovery
Added	Found	
4	3.6 ± 0.8	91 ± 19
8	8.6 ± 0.4	107 ± 5
15	14.0 ± 0.4	93 ± 3
20	20.0 ± 0.4	100 ± 2

* 3 replications

Table 37 Percentage recovery of *Salmonella* spiked in coconut juice (Local 2; 2nd sampling) using impedimetric biosensor system.

Concentration (cell/ml)		% Recovery
Added	Found	
4	3.7 ± 0.4	92 ± 12
8	9.4 ± 0.4	118 ± 6
15	14.4 ± 1.0	96 ± 8
20	20.4 ± 0.7	102 ± 4

* 3 replications

Table 38 Percentage recovery of *Salmonella* spiked in coconut juice (Local 2; 3rd sampling) using impedimetric biosensor system.

Concentration (cell/ml)		% Recovery
Added	Found	
4	3.8 ± 0.6	96 ± 17
8	8.4 ± 0.8	104 ± 13
15	16.0 ± 1.1	107 ± 9
20	19.6 ± 0.9	98 ± 5

* 3 replications

Table 39 Percentage recovery of *Salmonella* spiked in coconut juice (Local 2; 4th sampling) using impedimetric biosensor system.

Concentration (cell/ml)		% Recovery
Added	Found	
4	4.1 ± 0.4	103 ± 12
8	8.5 ± 0.7	106 ± 10
15	15.2 ± 1.5	102 ± 13
20	22.3 ± 1.2	111 ± 7

* 3 replications

Table 40 Percentage recovery of *Salmonella* spiked in orange juice (Commercial 3) using impedimetric biosensor system.

Concentration (cell/ml)		% Recovery
Added	Found	
4	4.0 ± 0.5	99 ± 15
8	8.9 ± 0.2	112 ± 4
15	14.0 ± 0.2	93 ± 2
20	18.5 ± 0.2	93 ± 1

* 3 replications

Table 41 Percentage recovery of *Salmonella* spiked in orange juice (Commercial 4; 1st sampling) using impedimetric biosensor system.

Concentration (cell/ml)		% Recovery
Added	Found	
4	4.3 ± 0.3	107 ± 8
8	8.4 ± 0.5	105 ± 7
15	13.6 ± 0	90 ± 0
20	18.7 ± 0.5	93 ± 3

* 3 replications

Table 42 Percentage recovery of *Salmonella* spiked in orange juice (Commercial 4; 2nd sampling) using impedimetric biosensor system.

Concentration (cell/ml)		% Recovery
Added	Found	
4	4.5 ± 0.4	113 ± 11
8	6.9 ± 0.4	86 ± 6
15	13.5 ± 1.3	90 ± 11
20	19.9 ± 1.1	99 ± 7

* 3 replications

Table 43 Percentage recovery of *Salmonella* spiked in orange juice (Commercial 4; 3rd sampling) using impedimetric biosensor system.

Concentration (cell/ml)		% Recovery
Added	Found	
4	4.4 ± 0.4	110 ± 13
8	8.1 ± 0.9	101 ± 13
15	15.4 ± 1.1	103 ± 9
20	20.0 ± 1.6	100 ± 10

* 3 replications

Table 44 Percentage recovery of *Salmonella* spiked in orange juice (Commercial 4; 4th sampling) using impedimetric biosensor system.

Concentration (cell/ml)		% Recovery
Added	Found	
4	3.3 ± 0.3	83 ± 9
8	8.8 ± 0.5	109 ± 8
15	15.3 ± 1.1	102 ± 9
20	19.5 ± 1.0	97 ± 6

* 3 replications

Table 45 Summary of percentage recovery of *Salmonella* spiked in sample.

Sample	% Recovery
Water (Commercial 1)	94 – 122
Water (Commercial 2)	101 – 129
Orange juice (Local 1)	79 – 114
Coconut juice (Local 2; 1st sampling)	91 – 107
Coconut juice (Local 2; 2nd sampling)	92 – 118
Coconut juice (Local 2; 3rd sampling)	96 - 107
Coconut juice (Local 2; 4th sampling)	102 – 111
Orange juice (Commercial 3)	93 – 112
Orange juice (Commercial 4; 1st sampling)	90 – 107
Orange juice (Commercial 4; 2nd sampling)	86 – 113
Orange juice (Commercial 4; 3rd sampling)	100 – 110
Orange juice (Commercial 4; 4th sampling)	83 – 109

* 3 replications