CHAPTER 2

RESEARCH METHODOLOGY

2.1 Materials

2.1.1 Biosensor

- Alumina polishing powder (Al_2O_3,: METKON, Turkey.)
- Hydrogen peroxide (H_2O_2,: Merck, Germany.)
- Sulfuric acid (H_2SO_4, AR Grade: LAB-SCAN, Thailand.)
- Potassium ferricyanide ($K_3Fe(CN)_6$,: Sigma, USA.)
- Potassium chloride (KCl, AR Grade: Univar, Australia.)
- Thioctic acid ($C_8H_{14}S_2O_2$,: Sigma, USA.)
- Ethanol (C_2H_5OH, Pro Analysis: Merck, Germany.)
- 1-ethyl-3-3(3-dimethylaminopropyl) carbodiimide hydrochloride ($C_8H_{17}N_3$ * HCl,: Sigma, USA.)
- Acetonitrile (CH_3CN,: Merck, Germany.)
- polyclonal antibody of *Salmonella* (IgG from goat,: BacTrace, USA.)
- 1-Dodecanethiol (Aldrich, USA.)
- Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$, AR Grade: Univar, Australia.)
- di-Sodium hydrogen orthophosphate dodecahydrate ($Na_2HPO_4.12H_2O$, AR Grade: Univar, Australia.)
- Sodium chloride (NaCl,: Carlo Erba, Italy.)
- Hydrochloric acid (HCl,: Scharlau, Spain.)
- Magnesium chloride dodecahydrate ($MgCl.12H_2O$,: Carlo Erba, Italy.)
- Glycine ($C_{12}H_{25}SH$, AR Grade: Riedel-de Haën, Germany.)
- Sodium hydroxide (NaOH,: LAB-SCAN, Thailand.)
- Potassium dihydrogenphosphate (K_2HPO_4,: AnalaR, UK.)
- di-Potassium hydrogen orthophosphate trihydrate ($K_2HPO_4.3H_2O$,: AnalaR, UK.)

- Tris(hydroxymethyl)aminomethane ($C_4H_{11}NO_3$, AR Grade: Fluka, Switzerland.)
- *Salmonella weltevreden* (WHO Salmonella-Shigella Center, Thailand.)
- *Shigella sonnei* (WHO Salmonella-Shigella Center, Thailand.)
- *E.coli* (WHO Salmonella-Shigella Center, Thailand.)

2.1.2 AOAC Official Method 967.25-967.27

- Buffered peptone water (Scharlau, Spain.)
- Selenite cystine (SC) broth (Difco, USA.)
- Bismuth sulfite (BS) agar (Merck, Germany.)
- SS agar (Merck, Germany.)
- Triple sugar iron (TSI) agar (Difco, USA.)
- MacConkey agar (Difco, USA.)
- Urea broth (Difco, USA.)
- Ducitol (Difco, USA.)
- Phenol red sucrose broth (BBL, USA.)
- Phenol red saccharose broth (Bacto, USA.)
- Tryptone broth (Bacto, USA.)
- Potassium cyanide (KCN,: Fisher Chemicals, UK.)
- Proteose peptone (Difco, USA.)
- Malonate broth (Bacto, USA.)
- *p*-Dimethylaminobenzaldehyde (Merck, Germany)
- Amyl alcohol ($CH_3(CH_2)_3CH_2OH$,: Carlo Erba, Italy.)
- Lactose (Difco, USA.)
- Sucrose ($C_{12}H_{22}O_{11}$, AR Grade, Univar, Australia.)
- MR-VP broth (Difco, USA.)
- Simmons citrate agar (BBL, USA.)
- Tryptic Soy agar (Difco, USA.)
- Tryptic Soy broth (Merck, Germany.)
- Plate count agar (Difco, USA.)

2.2 Equipments

2.2.1 Biosensor

- Polishing machine (Gripo® 2V, Metkon Instruments Ltd., Turkey.)
- Ultrasonicator ()
- Autolab (Ecochemie, Utrecht, Netherlands)
- Peristaltic pump (Minipulse3, Gilson, France.)
- Sample injector (VIGO 6 port W valves, Valco, USA.)
- Ultrasonicator probe (Branson Sonifier 450, Branson Ultrasonics, USA.)

2.2.2 AOAC Official Method 967.25-967.27

- Incubator (Gallenkamp, UK.)
- Laminar air flow (Astec microflow, Bioquell Medical Ltd., UK.)

2.3 Impedimetric immunosensor detection principle

An impedimetric immunosensor to detect *Salmonella* was developed by immobilizing polyclonal antibody against *Salmonella* on gold working electrode via self-assembled monolayer (SAM) (see 2.4.2). Impedance measurements were performed in a three-electrode electrochemical system as shown in Figure 7. An Autolab PGSTAT30 equipped with a FRA2 module controlled by FRA 4.9 was used for the impedance measurement.

A flow injection impedimetric biosensor system is shown in Figure 8 and consisted of

a) A peristaltic pump, where steady flow rate of the solution was controlled.

b) An injection valve where a specific volume of the sample was injected into the analysis system through the sample carrier buffer.

c) A detection unit consisted of a reaction flow cell (Figure 7) and an Autolab that monitored the change in the impedance resulting from the binding of antibody against *Salmonella* with *Salmonella.* The flow cell consisted of three electrodes, i.e., a modified working electrode with immobilized anti-*Salmonella*, a Ag/AgCl reference electrode and a stainless steel auxiliary electrode. An autolab was

used to record, display and analyze experimental data. When solution containing *Salmonella* was injected into the flow system, the binding between antigens and antibodies caused the impedance to increase and this was related to the amount of *Salmonella*.

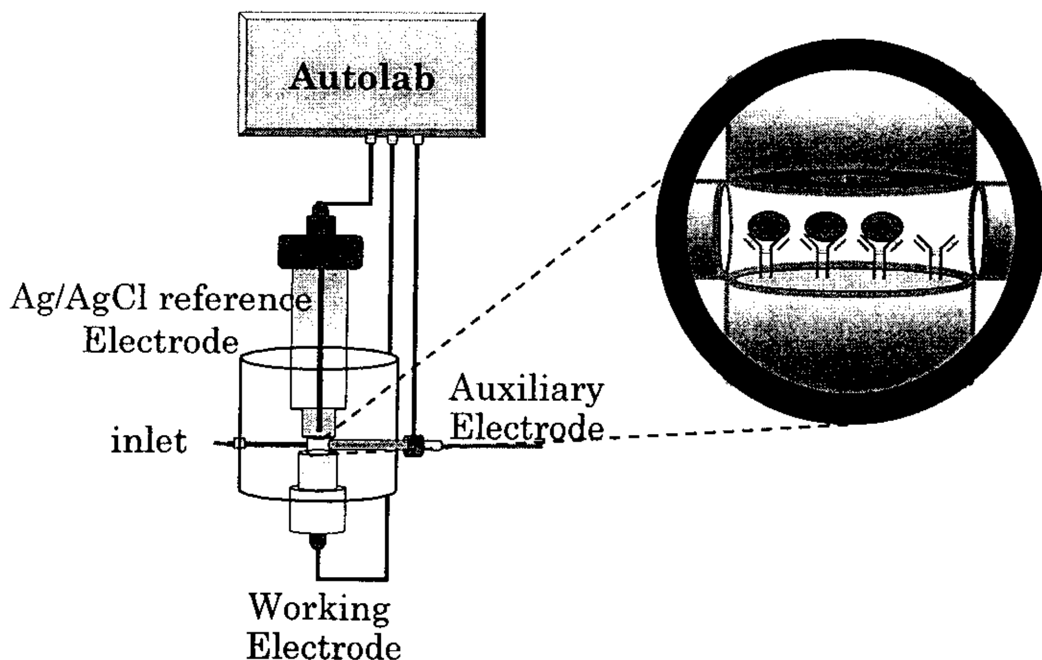


Figure 7 : Schematic diagram showing reaction flow cell.

(Modified from Limbut, 2006)

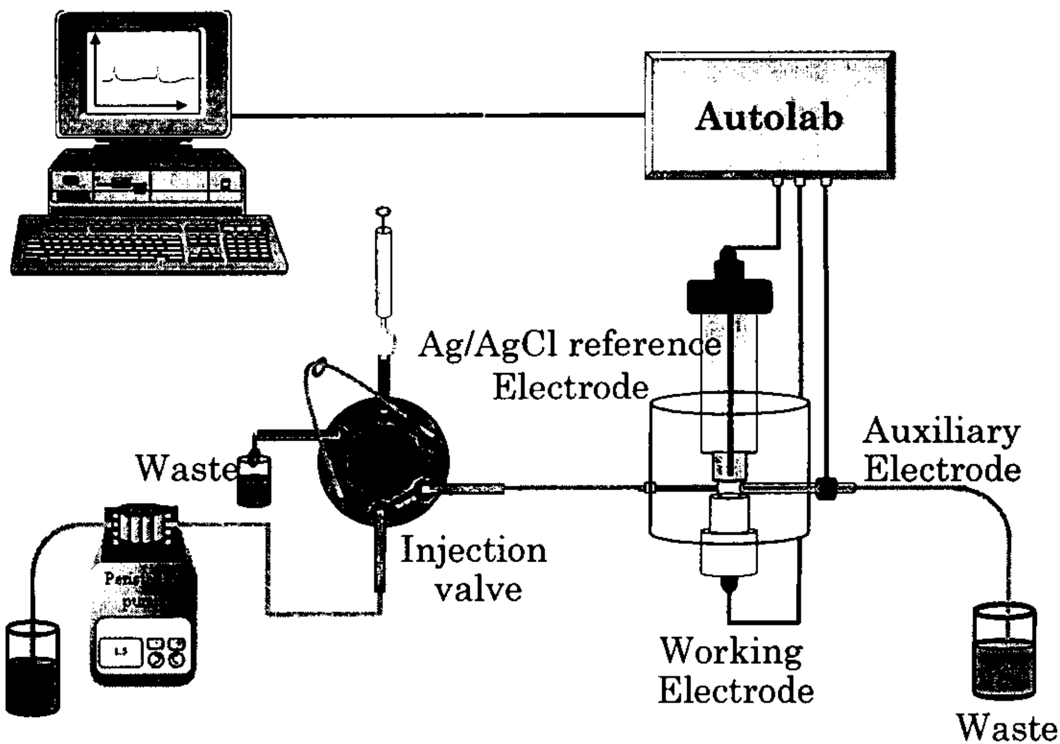


Figure 8 : Schematic diagram showing the impedemetric immunosensor system.
(Modified from Limbut, 2006)

2.4 Modified working electrode

2.4.1 Preparation of gold electrode

Gold electrodes (∅ 3 mm, 99.9% purity) were sonicated with piranha reagent and polished with alumina slurries 5, 1 and 0.3 μm, respectively, using a polishing machine and then cleaned through sonication consequentially in absolute ethanol and distilled water for 15 min. Each electrode was dried with pure nitrogen gas and was electrochemically cleaned in 0.5 M H_2SO_4 by cycling the potential over the range 0 to 1.4 V *vs* Ag/AgCl reference electrode with a scan rate of 100 mV s^{-1} until a stable voltammogram was obtained.

2.4.2 Immobilization

A cleaned gold electrode was immersed in 250 mM of thioctic acid in absolute ethanol at room temperature for 12 hours. After thoroughly rinsed with absolute ethanol and dried with pure nitrogen gas, the modified electrode was immersed in 1% (w/w) of EDC in acetonitrile for 5-6 hours to activate the free carboxylic groups of thioctic acid (Akram *et al*, 2004). Then the electrode was rinsed with 100 mM potassium phosphate buffer saline (i.e., with 0.9% w/v of NaCl) pH 7.4 and dried with pure nitrogen gas. Anti-*Salmonella*, 25 μl of 0.1 mg ml^{-1}, was spread over the surface of the modified electrode and kept in a refrigerator at 4°C overnight. Finally, 10 mM of 1-dodecanethiol ethanoic solution was used to block pinholes on the electrode surface for 25 – 30 minutes before placing in the flow-cell. Figure 9 shows the reaction mechanism during the immobilization steps.

An Autolab PGSTAT10 with GPES 4.9 software was used to investigate the polishing and modification steps of the electrode. Cyclic voltammetric measurement was performed in an unstirred solution of permeable redox couple (*i.e.* 5 mM $K_3[Fe(CN)_6]$ containing 0.1 M KCl) using an electrochemical cell equipped with three electrode system consisted of the immobilized anti-*Salmonella* gold electrode as working electrode, an Ag/AgCl reference electrode and a platinum wire as auxiliary electrode (Figure 10). Potential was scanned from 0 to 1.4 V at a scan rate of 100 mV/s.

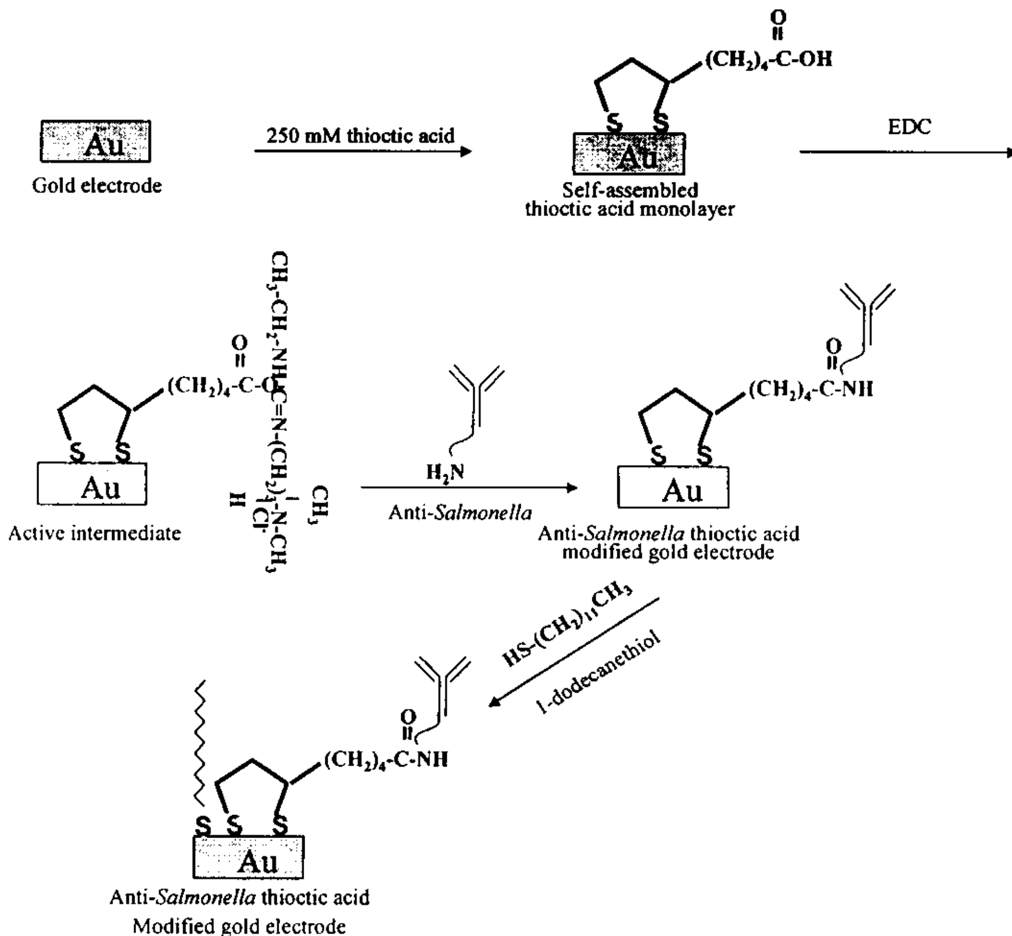


Figure 9 : Reaction mechanism of the immobilization steps of anti-*Salmonella* on a self-assemble thioctic acid monolayer (Modified from Limbut, 2006)

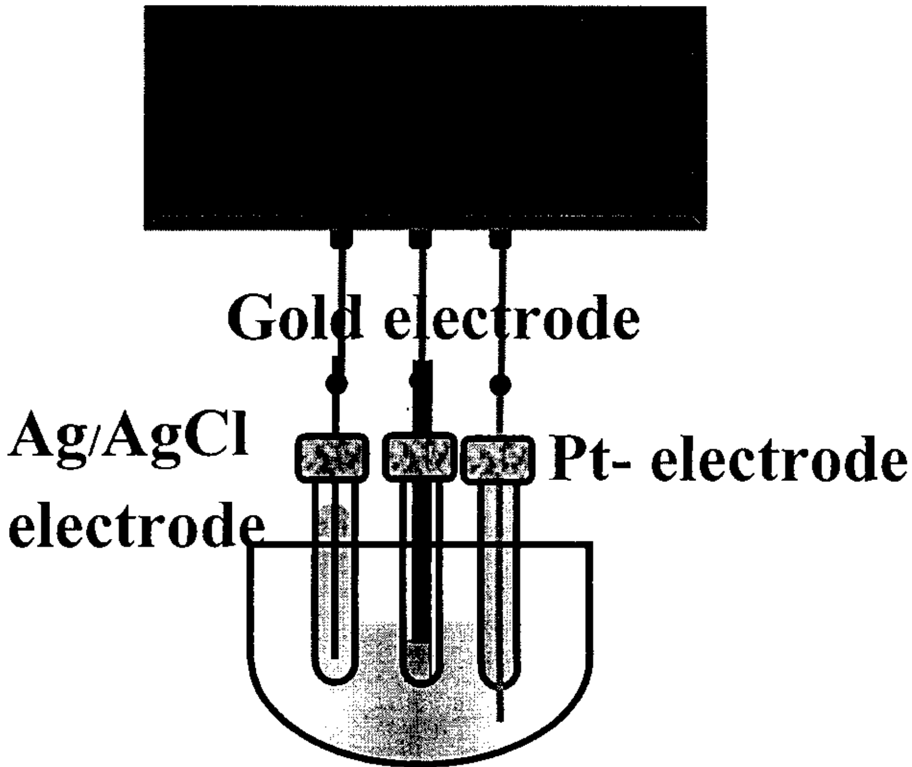


Figure 10 : Schematic diagram showing the cyclic voltammetry system.
(Modified from Limbut, 2006)

2.5 Microbiology method

2.5.1 Preparation of media

Distilled water was used to prepare all media which came in powder form.

2.5.1.1 Tryptic Soy broth

Composed of (g l^{-1}): trypticase peptone 17, phytone peptone 3, NaCl 5, K_2HPO_4 2.5 and glucose 2.5. The mixture was heated with gentle agitation until all components were dissolved. Then 225 ml of the mixture was dispensed into 500 ml Erlenmeyer flasks and capped with cotton wool. Autoclaved at 121 °C for 15 min.

2.5.1.2 Plate count agar

Composed of (g l^{-1}): tryptone 5, yeast extract 2.5, dextrose 1 and agar 15. The mixture was heated to dissolve the ingredients. Dispensed into suitable capped tubes or flasks capped with cotton wool. Autoclaved at 121 °C for 15 min.

2.5.1.3 Buffered peptone water broth

Composed of (g l^{-1}): peptone 10, NaCl 5, Na_2HPO_4 3.5 and KH_2PO_4 1.5. Dispensed into suitable capped tubes or flasks capped with cotton wool. Autoclaved at 121 °C for 15 min.

2.5.1.4 Selenite cystine broth

Composed of (g l^{-1}): tryptone 5, Lactose 4, $NaHSeO_3$ 4, Na_2HPO_4 10 and L-Cystine 0.01. The mixture was heated until boiling to dissolve the ingredients. Then 10 ml of the portions was dispensed into sterile 16 x 150 mm test tubes and heated for another 10 min in boiling steam. The broth could only be used on the day of preparation.

2.5.1.5 Bismuth sulfite agar

Composed of (g l^{-1}): peptone 10, beef extract 5, dextrose 5, Na_2HPO_4 4, $FeSO_4$ 0.3, bismuth sulfite 8, brilliant green 0.025 and agar 20. The mixture was mixed thoroughly and heated with agitation. Boiled about 1 min to obtain uniform suspension (there will be some precipitate that could not dissolve). Cooled to 45-50 °C. Suspended the precipitate by gentle agitation and poured 20 ml portions into sterile Petri dish. Let plates dry about 2 h with lids partially removed then closed plates. The plates were prepared 1 day before used and stored in the dark.

2.5.1.6 Triple sugar iron slant

Composed of (g l^{-1}): typticase peptone 17, phytone peptone 3, NaCl 5, K_2HPO_4 2.5 and glucose 2.5. The mixture was heated with gentle agitation until all

components were dissolved. Then 225 ml of the mixture was dispensed into 500 ml Erlenmeyer flask and capped with cotton wool. Autoclaved at 121 °C for 15 min.

2.5.1.7 MacConkey agar

Composed of (g l^{-1}): proteose peptone 3, peptone 17, lactose 10, bile salts No.3, NaCl 5, Neutral red 0.03, crystal violet 0.001and agar 13.5. The mixture was suspended and heated with agitation until all components were dissolved. Boiled for 1-2 min. Autoclaved at 121 °C for 15 min, cooled to 45-50 °C and poured 20 ml portions into sterile 15 x 100 mm Petri dishes. Dried at room temperature with lids closed.

2.5.1.8 Urea broth

Composed of (g l^{-1}): urea 20, yeast extract 0.1, Na_2HPO_4 9.5, K_2HPO_4 9.1 and phenol red 0.01. The mixture was sterilized by filtration through 0.45 μm membrane. Then 1.5-3.0 ml was aseptically dispensed into 13 x 100 mm sterile test tubes.

2.5.1.9 Phenol red lactose broth

Composed of (g l^{-1}): trypticase 10, NaCl 5, beef extract 1, phenol red 0.017 and lactose 10. Then 2.5 ml was dispensed into 13 x 100 mm capped tubes. Autoclaved at 118 °C for 10 min.

2.5.1.10 Phenol red sucrose broth

Composed of (g l^{-1}): trypticase 10, NaCl 5, beef extract 1, phenol red 0.017 and sucrose 10. Then 2.5 ml was dispensed into 13 x 100 mm capped tubes. Autoclaved at 118 °C for 10 min.

2.5.1.11 Simmons citrate

Composed of (g l^{-1}): Sodium citrate 2, NaCl 5, K_2HPO_4, $NH_4H_2PO_4$ 1, $MgSO_4$ 0.2, Bromthymol blue 0.08 and agar 15. The mixture was heated gently with occasional agitation. Boiled for 1-2 min until agar dissolves. Filled 13 x 100 or 16 x 150 mm screw-cap tubes 1/3 full. Autoclaved at 121 °C for 15 min. Before medium solidified, inclined tubes to obtain 4-5 cm slants and 2-3 cm butts.

2.5.1.12 Phenol red ducitol broth

Composed of (g l^{-1}): trypticase 10, NaCl 5, beef extract 1, phenol red 0.017 and ducitol 5. Then 2.5 ml was dispensed into 13 x 100 mm capped tubes. Autoclaved at 118 °C for 10 min.

2.5.1.13 Potassium cyanide broth

Composed of (g l^{-1}): KCN 0.5, Proteose peptone 3, NaCl 5, KH_2PO_4 0.225 and Na_2HPO_4 5.64. The mixture was dissolved above ingredients except KCN and autoclaved 15 min at 121 °C. Cooled and refrigerated at 5-8 °C. Prepared KCN solution by dissolving 0.5 g KCN in 100 ml sterile distilled water cooled to 5-8 °C. Added 15 ml cold KCN stock solution using bulb pipetter to 1 liter of above mixture. Mixed and aseptically dispensed 1.0-1.5 ml portions to 13 x 100 mm sterile tubes. Using aseptic techniques, stopper tubes with No. corks impregnated with paraffin. Prepare corks by boiling in paraffin about 5 min. Placed corks in tubes so that paraffin did not flow into broth but formed a seal between rim of tubes and cork. Stored tubes at 5-8 °C no longer than 2 weeks before used.

2.5.1.14 Kovacs' reagent

Dissolved *p*-dimethylaminobenzaldehyde 5 g in normal amyl alcohol 75 ml. Slowly added con. HCL 25 ml. Stored at 4 °C.

2.5.1.15 Malonate broth

Composed of (g l^{-1}): yeast extract 1, $(NH_4)_2SO_4$ 2, K_2HPO_4 0.6, KH_2PO_4 0.1, NaCl 2, Sodium malonate 3, glucose 0.25 and bromthymol blue 0.025. The mixture was dissolved by heating if necessary. Then 3 ml was dispensed into 13 x 100 mm capped test tubes. Autoclaved at 121 °C for 15 min.

2.5.1.16 MR-VP broth

Composed of (g l^{-1}): buffered peptone water powder 7, glucose 5 and K_2HPO_4 5. The mixture was dissolved in water with gentle heat if necessary. Then 10 ml was dispensed into 16 x 150 mm capped test tubes and autoclaved at 121 °C for 15 min.

2.5.2 Microbiology technique

2.5.2.1 Aseptic Technique

Aseptic technique is used to prevent the contamination during manipulations of cultures and sterile culture media. It involves avoiding any contact of the pure culture, sterile medium and sterile surfaces of the growth vessel with contaminating microorganisms. The steps for transferring the culture from one vessel to another are shown in Figure 11. Essentially the same technique is used for inoculating Petri dishes, except that the dish is not flamed and for transferring microorganisms from a culture vessel to a microscope slide.

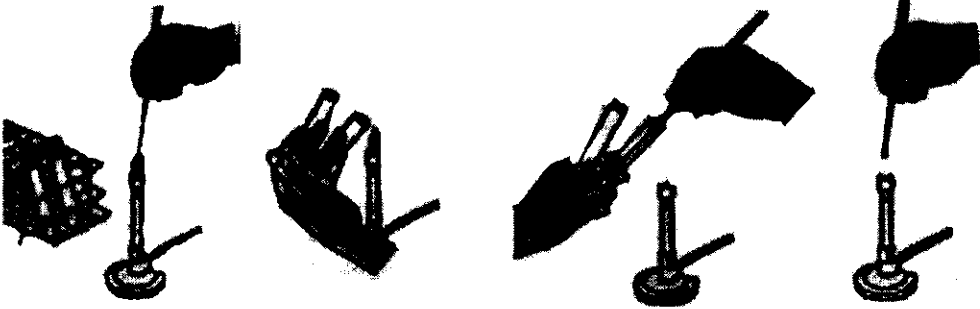


Figure 11 : Steps in the aseptic transfer of bacteria (Modified from Atlas, 1995)

2.5.2.2 Streak plate

In the streak plate technique for isolating pure cultures of bacteria, a loopful of bacterial cells is streaked across the surface of a sterile solidified agar plate that contains a nutrient medium. The steps for streaking plate are shown in Figure 12. The plate is then incubated under favorable conditions to permit the growth of the bacteria.

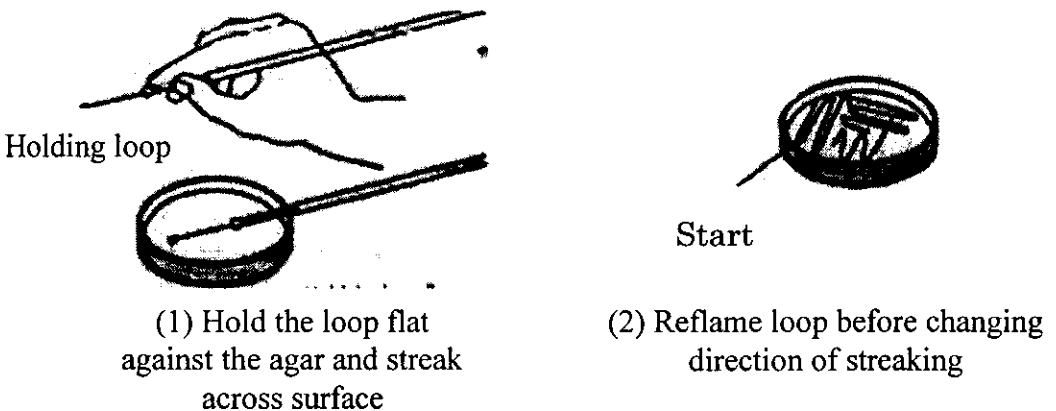
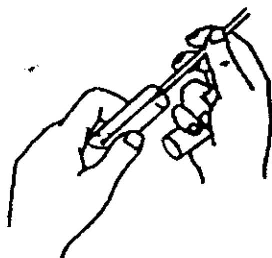
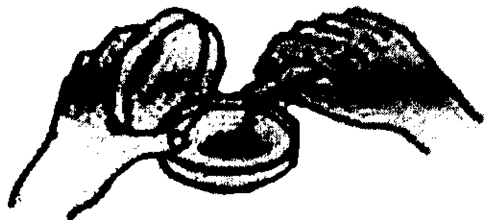


Figure 12 : Steps in the streak plate technique (Modified from Atlas, 1995)

2.5.2.3 Pour plate

The pour plate technique is used for the quantification of numbers of viable bacteria. Suspensions of microorganisms are added to tubes containing melted agar cooled to approximately 42 to 45 °C. The bacteria and agar medium are mixed well and the suspensions are poured into sterile Petri dishes using aseptic technique. The agar is allowed to solidify, trapping the bacteria at separate discrete positions within the medium. Figure 13 shows the steps of pour plate technique.





(1) Use loop or pipette add bacterial suspension to dilution in melted agar

(2) pour

Figure 13 : The steps of pour plate technique
(Modified from Atlas, 1995; Brock and Madigan, 1991)

2.6 Bacteria and culture plating method

Pure culture bacteria, *i.e.*, *Salmonella weltevreden* the bacteria which is the most found in food (The National *Salmonella* and *Shigella* Center, 2004), *E.coli* and *Shigella sonnei* were obtained from the National *Salonella* and *Shigella* Center, National Institute of Health, Thailand. They were cultured separately in 30 ml of Tryptic Soy broth in a sterilized tube and incubated in an incubator at 35°C for 24 h before use.

The bacterial concentration was determined by the conventional dilution plate count method. The bacteria culture was diluted serially using 0.85% (w/v) NaCl. One milliliter of each serial dilution was transferred with a sterile pipette into 20 ml of the melted plate count agar and then mixed well. The mixture was then poured into a sterile Petri dish then let the mixture became solid. The Petri dishes

were incubated in the incubator at 35 °C for 24 h. The concentration of bacteria was then counted by a colony counter.

Before the culture in the broth was used in the biosensor system, the culture was heated at 100°C for 15 min to kill all the bacteria in order to stop their growth and diluted to the desired concentrations with 100 mM of phosphate buffer saline for further use.

2.7 Impedimetric measurement

An Autolab PGSTAT30 equipped with a FRA2 module controlled by FRA 4.9 was used for the impedance measurement. For quantitative analysis, it is appropriate to measure changes of the impedance at a single frequency. Since the impedance depends on the frequency (Bart *at al.*, 2005) an optimum frequency needs to be studied. The dependence of impedance on frequency can be investigated using a Bode plot (Navrátiloá and Skládal, 2004) (Figure 14). The optimum frequency was determined by considering the plot between phase angle and logarithm of impedance vs. the logarithm of frequency as shown in Figure 14. The optimum frequency is chosen in the region where the plot of impedance vs. log frequency is a straight line with a slope of -1 and the phase angle closest to -90° (Wu *et al.*, 2005)

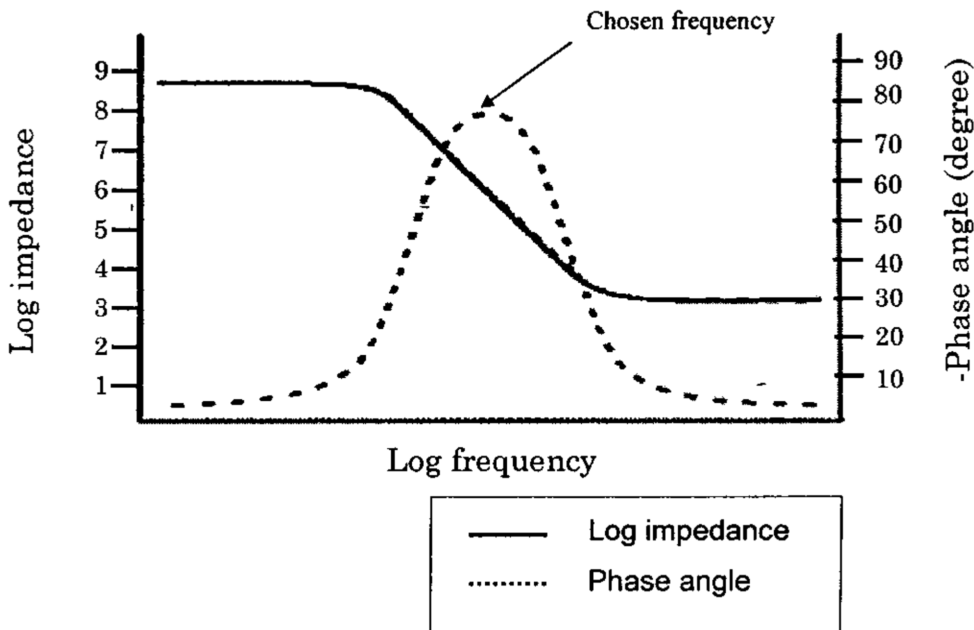


Figure 14 : Schematic diagram of the Bode plot (Modified from Lillie *et al.*, 2001)

Once the optimum frequency was obtained it was used to monitor the change of impedance with time. After the injected solution passed through the modified electrode in the flow cell, both components of the impedance, i.e., Z' and Z" increased with respect to the steady state impedance before analyte injection (the baseline) but the increase of Z' did not correlate well with the antigen concentration (Bart *et al.*, 2005), therefore, only Z" was investigated in further analysis as shown in Figure 15.

The detection is based on the change in the impedance after antigen-antibody reaction which was taken as

$$\Delta Z'' = Z''_{AgAb} - Z''_{Ab}$$

Where Z''_{Ab} is the value of the steady-state impedance of the immobilized antibody in a buffer solution, and Z''_{AgAb} represents a value of the steady-state impedance after the antigen-antibody reaction under the same conditions.

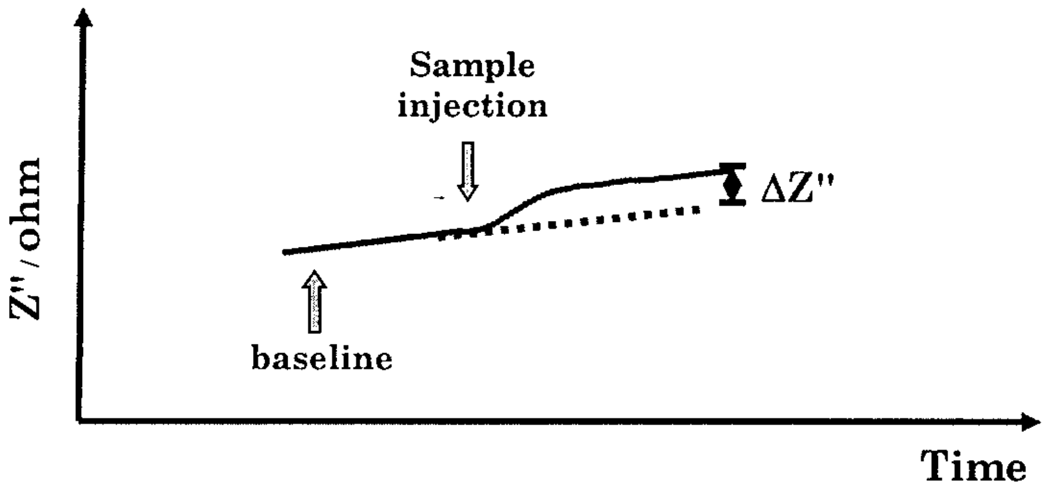


Figure 15 : Schematic diagram showing the change in impedance (ΔZ") as a function of time caused by binding between antigen and antibody

2.8 Optimization of operational conditions

Experimental parameters affecting the performance of the system were investigated and optimized to yield the highest signal. These include type and concentration of regeneration solution, sample volume, flow rate, type of buffer, pH of buffer and concentration of buffer. In these studies, 100 cell/ml of *Salmonella weltevreden* in 10 mM phosphate buffer pH 7.40, a concentration in the linear dynamic range, was injected. The measurements were performed in triplicate by changing a single variable while the others were kept constant. When an optimum condition was obtained, it was used to optimize the next parameter. The starting operational conditions are shown in Table 2.

Table 2 Starting operational conditions.

Condition	Value
1. Type of regeneration solution	Low pH : HCl
2. Concentration of regeneration solution (mM)	3.16 mM (pH 2.5)
3. Sample volume (μl)	250
4. Flow rate (μl/min)	250
5. Type of buffer	Sodium phosphate buffer saline
6. pH of buffer	7.40
7. Concentration of buffer (mM)	10

2.8.1 Type of regeneration solution

An important issue for immunosensors is the question of whether the surface can be regenerated without significant loss of activity (Thévenot, 2001). The dissociation of the target analyte-biorecognition element complex is possible by using regeneration solution. Regeneration allows surfaces to be reused many times, saving both time and money (van der Merwe, 2000).

The regeneration solutions tested in this work were divided into three categories. The concentration and types of solutions investigated were, i.e. hight ionic strength (Pei *et al.*, 2000), low pH (Chou *et al.*, 2002) and high pH (Park *et al.*, 2000)

1) high ionic strength: 1M NaCl, 1M KCl and 1M $MgCl_2$

2) low pH: Glycine/HCl pH 2.5, HCl pH 2.5 and HCl pH 2.0

3) high pH: 5 mM NaOH and 50mM NaOH

In order to investigate the optimum regeneration solution, starting operational conditions 2-6 in Table 2 were used. Standard solution of 100 cell/ml of *Salmonella* was injected into the biosensor system during a continuous flow of buffer. The binding between *Salmonella* and the immobilized antibody caused an increase in the signal (Figure 16). Regeneration was done after the signal was constant by injecting the regeneration solution as shown in Figure 16. This step was done in triplicate. Then other regeneration solutions were tested using the same procedure.

The optimum regeneration solution was evaluated by considering the residual activity of the modified electrode after regeneration. The residual activity can be calculated from the impedance change ($\Delta Z''$) resulting from the binding between antigen-antibody of *Salmonella* before ($\Delta Z''_1$) and after regeneration ($\Delta Z''_2$)

$$\% \text{ residual activity} = \frac{\Delta Z''_2 \times 100}{\Delta Z''_1}$$

Figure 16 : Schematic diagram showing the change in impedance ($\Delta Z''$) as a function of time causes by binding between antigen and antibody with subsequent signal decrease due to dissociation under regeneration conditions.

The criteria for regenerating electrode surface is that if post-regeneration binding remains above 90% compared to the binding efficiency before regeneration, the used conditions should be seen as adequate (van der Merwe, 2000).

2.8.2 Concentration of regeneration solution

Regeneration conditions could affect the performance and the lifetime of the electrode because when the antigens bound to the antibodies on the electrode it caused the impedance to increase, then after each analysis, the antigens need to be removed in order to be able to be used again. If the conditions of the regeneration solutions are not suitable, the antibodies or the layers can be destroyed. After the type of regeneration solution was obtained, the concentration of the regeneration solution

was also tested in order to approach higher % residual activity. The effect of the concentration solution was tested at 5, 10, 15, 20, 25, 50, 100 and 200 mM of NaOH.

2.8.3 Sample volume

One way to improve the response of the system is to increase analyte by increasing the sample volume. The effect of the sample volume was studied at 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 μl.

2.8.4 Flow rate

In a flow system, variations in flow rate revealed differences in the binding responses of the antibodies to the antigen. The effect of the flow rate was studied at 50, 100, 150, 200, 250, 300, 350 and 400 μl/min.

2.8.5 Type of buffer

It is necessary to find the type of buffer solution which is best suited for the impedimetric immunosensor system. The buffers that have been used in the impedimetric immunosensor were 10 mM phosphate buffer saline pH 7.4 (composed of 10 mM phosphate buffer, 2.7 mM KCl and 137 mM NaCl) (Ouerghi *et al.*, 2002), 100 mM phosphate buffer saline pH 7.2 (contains $K_3Fe(CN)_6/K_4Fe(CN)_6$) (Diniz *et al.*, 2003) and 10 mM Tris-HCl pH 7.4 (McNeil *et al.*, 1995). The influence of type of buffer was then tested using 10 mM of sodium phosphate buffer, potassium phosphate buffer and Tris-HCl buffer. From the preliminary study, phosphate buffer containing $K_3Fe(CN)_6/K_4Fe(CN)_6$ clogged the reference electrode, therefore, only sodium phosphate buffer saline, potassium phosphate buffer saline and Tris-HCl were investigated.

2.8.6 pH of buffer

The pH of buffer that have been used in the immunosensor for *Salmonella* detection were 10 mM phosphate buffer saline pH 7.4 (Su and Li, 2005),

50 mM phosphate buffer saline pH 7.2 (Wong *et al.*, 2002). In this work, the effect of pH of phosphate buffer saline was investigated at pH 6.8, 7.0, 7.2, 7.4, 7.6, 7.8 and 8.0.

2.8.7 Concentration of buffer

The effect of ionic strength of buffer solution corresponds to concentration of buffer (Bezerra *et al.*, 2003). The effect of concentration of phosphate buffer saline was evaluated at 5, 10, 25, 50, 100, 200 and 400 mM.

The optimized parameters and values are summarized in Table 3

Table 3 Optimized parameters and values of the operational conditions

Parameters	Optimized values
1. Type of regeneration solution	ionic strength, low pH and high pH
2. Concentration of regeneration solution (mM)	5, 10, 15, 20, 25, 50, 100 and 200
3. Sample volume (μl)	50, 100, 150, 200, 250, 300, 350, 400, 450 and 500
4. Flow rate (μl/min)	50, 100, 150, 200, 250, 300, 350 and 400
5. Type of buffer	Sodium phosphate buffer saline, potassium phosphate buffer saline and Tris-HCl
6. pH of buffer	6.8, 7.0, 7.2, 7.4, 7.6, 7.8 and 8.0
7. Concentration of buffer (mM)	5, 10, 25, 50, 100 and 200

2.9 System performance

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

2.9.1 Limit of detection

The limit of detection is defined as the amount or the content of an analyte corresponding to the lowest measurement signal which with a certain statistical confidence maybe interpreted as indicating that the analyte is present in the solution/analytical sample, but not necessarily allowing exact quantification (AOAC, 2004). Limit of detection can be calculated using following equation.

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

Where LOD = the limit of detection

S_a = the standard deviation of x blank results ($x > 20$)

b = the slope of the calibration curve/regression line

100 mM phosphate buffer saline pH 7.4 (blank) were injected into biosensor system for twenty times under the optimum conditions. The standard deviation was calculated. The slope of the calibration curve was determined from the calibration curve obtained from the injection of standard solutions of *Salmonella* at concentrations between 1 cell/ml and 1400 cell/ml into the biosensor system in triplicate under the optimum conditions. The limit of detection was then calculated using the above equation.

2.9.2 Linearity

The linear range is defined as the concentration range over which the intensity of the signal obtained is directly proportional to the concentration of the species producing the signal (IUPAC, 1997)

Standard solutions of *Salmonella* in the range of 1 cell/ml to 600 cell/ml were injected into the biosensor system under the optimum conditions. The linearity of the response was determined by plotting the calibration curve. The linearity of the response was determined by considering the linear regression coefficient.

2.9.3 Stability of immobilized electrode

All organic materials deteriorate with times, especially when taken out of their natural environment. This means that one of the major drawbacks with biosensors is that the biological component usually has a fairly limited lifetime before it need replacing (Eggins, 1996).

The stability of the modified electrode was studied. Under optimum conditions, 100 cell/ml of standard solutions of *Salmonella* were injected into the biosensor system and regenerate using the optimized regeneration solution until the response of the biosensor system gave the % residual activity less than 90%. The stability of the immobilized electrode can be shown as the number of the injection which can indicate the number of the reused.

2.9.4 Selectivity

Selectivity gives the reliability to the developed analytical method. The selectivity was tested by using different substrates in which their physical or chemical characteristics are similar to the target analytes. For this work *E.coli* and *Shigella sonnei*, the same pathogenic bacteria in family *Enterobacteriaceae* as *Salmonella*, were used. Standard solutions of *E.coli* and *Shigella sonnei* in the concentration of 4, 8, 15 and 25 cell/ml were injected (in triplicate) into the biosensor system under the optimum conditions. The calibration curve of each bacteria was plotted and compared to the calibration curve of *Salmonella weltevreden.*

In this work, *Salmonella weltevreden* was chosen to be the representation of *Salmonella* spp. because it is the serovars that found the most in water of Thailand (41.49%). It is also found the most in all types of samples in southern region of Thailand (27.86%) (The National *Salmonella* and *Shigella* Center, 2004).

Another reason is that the anti-Salmonella used in this work are affinity-purified *caprine* polyclonal antibodies that can recognize and bind to all *Salmonella* serovars available from the American Type Culture Collection obtained from Kirkegaard and Perry Laboratories (Gaithersburg, USA). These antibodies have been used in many researches to detect all *Salmonella* serovars. Generally, only some

serovars would be used as the representative for the detection of *Salmonella* spp. e.g., *Salmonella typhimurium* (Park *et al.*, 2000; Abdel-Hamid *et al.*, 1999), *Salmonella enteritidis* (Brovko *et al.*, 2004). Bokken *et al.* (2003) detected 53 *Salmonella* serovars using an optical surface plasmon resonance biosensor and showed the ability of these antibodies in detecting all *Salmonella* serovars. Therefore, *Salmonella weltevreden* was the species chosen to be the representative in this work.

2.9.5 Activity of homogenized cell

In order to test the efficiency of the biosensor system whether it can be used to detect the antigen of the bacterial cell when the cell is disrupted, the culture was homogenized by using the ultrasonicator probe at the duty cycle % constant of 40, output control at 40, time cycle at 30 s for 30 minute before injected through the system.

2.10 Determination of Salmonella in juices

To demonstrate the use of the developed impedimetric biosensor, the system was tested using samples obtained from many sources in Hat Yai, Songkhla. These samples were analyzed by the impedimetric biosensor and AOAC Official Method 967.26

2.10.1 Impedimetric biosensor system

To calibrate the system, standard solution of *Salmonella* at 4, 8, 15 and 20 cell/ml were tested under the optimum conditions. The calibration curve was prepared by plotting the impedance change vs. the concentration of *Salmonella*. The sample solutions were then injected into the system. The change in the impedance of each sample was used to calculate the concentration of *Salmonella* from the calibration curve which was done prior the test. The experiments were done in triplicate and the average sensor response and standard deviation (S.D.) were calculated.

2.10.2 Impedimetric biosensor system using standard addition method

When analyzing real samples, matrix interference or matrix effect may play an important role in the accuracy and precision of a measurement and the matrix effect was investigated. Matrix spikes was used to determine the effect on sample signal. Spiking known amount of *Samonella* standard solution to the sample and diluted with 10 mM phosphate buffer saline pH 7.4 to the concentration of 4, 8, 15 and 20 cell/ml. The sample with no *Salmonella* (sample blank) was also analyzed. A 400 μl aliquot of each solution was injected into the biosensor system under the optimum conditions. Each experiment was done in triplicate. The impedance changes were plotted against the concentrations. The slope of the standard and spiked samples were compared for matrix interference and evaluated using statistical test.

2.10.3 AOAC Official Method 967.26

Twenty five milliliters of each sample was aseptically added to 225 ml of buffered peptone water broth and incubated for 24 $\pm$ 2 h at 35°C. Then 0.1 ml of the mixture was transferred to 10 ml selenite cystine broth, mixed and incubated for 24 $\pm$ 2 h at 35°C. Streaked 3 mm loopful on the incubated selenite cystine broth on bismuth sulfite agar then incubated plates for 24 $\pm$ 2 h at 35°C.

If brown, gray, or black colonies were present on bismuth sulfite agar, then pick 2 or more colonies, lightly touched the very center of the colony with sterile inoculating needle, and streaked the colony into triple sugar iron slant then inoculated. *Salmonella* in culture typically produces alkaline (red) slant and acid (yellow) butt, with or without production of H_2S (blackening of agar) in triple sugar iron.

In order to identify *Salmonella*, for the mixed cultures (the cultures consisting of more than one type of microorganism), streaked positive colony from triple sugar agar on MacConkey agar then incubated plate for 24 $\pm$ 2 h at 35°C. Typical colonies appear transparent and colorless. For pure cultures, culture from triple sugar agar was inoculated with sterile needle into tubes of urea broth and incubated for 24 $\pm$ 2 h at 35°C. Typical colonies doesn't change the color.

To classify as *Salmonella*, the cultures exhibit typical *Salmonella* reaction for the tests as shown in Table 4.

Table 4 Biochemical reactions of *Salmonella* (U.S. Food and Drug Administration, 2005)

Biochemical test	Result		*Salmonella* species reaction
	Positive test	Negative test	
Phenol red lactose broth	Yellow color and/or gas	No gas / no color change	-(c)
Phenol red sucrose broth	Yellow color and/or gas	No gas / no color change	-(a)
Simmons citrate	Growth, blue color	No growth, no color change	V
Phenol red ducitol broth	Yellow color and/or gas	No gas / no color change	+(b)
KCN broth	Growth	Not growth	-(a)
Indole test	Violet color at surface	Yellow color at surface	-(a)
Malonate broth	Blue color	No color change	- (c)
Voges-Proskauer test	Pink to red color	No color change	-(a)
Methyl red test	diffuse red color	Diffuse yellow color	+(a)

a +, 90% or more positive in 1 or 2 days; -, 90% or more negative in 1 or 2 days; v, variable.

b Majority of *S. arizonae* cultures are negative.

c Majority of *S. arizonae* cultures are positive.