Impedimetric Immunosensor for Penicillin G

Supaporn Dawan

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Author	Miss Supaporn Dawan
Major Program	Analytical Chemistry

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ชื่อวิทยานิพนธ์	อิมพีดิเมตริกอิมมูโนเซนเซอร์สำหรับเพนนิซิลินจี
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บทคัดย่อ

พัฒนาระบบโฟลว์อินเจคชันอิมพิดิเมตริกอิมมูโนเซนเซอร์ (flow injection impedimetric immunosensor) เพื่อตรวจวัดปริมาณเพนนิซิลินจี (penicillin G) ในน้ำนม โดยตรึงแอนติเพนนิซิลินจี (anti-Penicillin G) บนเซลฟ์-แอสเซมเบิลกรดไทออกติกโมโนเลเยอร์ (self-assembled thioctic acid monolayer) บนขั้วอิเล็กโทรดทองทำงาน (gold working electrode) การจับกันอย่างจำเพาะเจาะจงของเพนนิซิลินจีและแอนติเพนนิซิลินจีบนขั้วอิเล็กโทรดทำงานทำให้ค่าอิมพีแดนซ์ (impedance) เพิ่มขึ้น วัดค่าอิมพีแดนซ์อย่างต่อเนื่อง ณ ความถี่ที่ให้สัญญาณการตอบสนองสูงสุด ที่ 160 เฮิรตซ์ ภายใต้สภาวะที่เหมาะสมระบบมีช่วงความเป็นเส้นตรงระหว่าง 1.0×10^{-13} ถึง 1.0×10^{-8} โมลาร์ และมีค่าขีดจำกัดต่ำสุดของการตรวจวัดอยู่ที่ 3.0×10^{-15} โมลาร์ ต่ำกว่าค่าสูงสุดที่อนุญาตให้เกิดการตกค้างได้ (1.0×10^{-8} โมลาร์) โดยใช้เวลาในการวิเคราะห์ 10-12 นาที แอนติเพนนิซิลินจีที่ตรึงบนเซลฟ์-แอสเซมเบิลกรดไทออกติกโมโนเลเยอร์บนหนึ่งอิเล็กโทรดสามารถใช้วิเคราะห์ได้ถึง 45 ครั้ง โดยมีค่าเบี่ยงเบนมาตรฐานสัมพัทธ์ (relative standard deviation, RSD) น้อยกว่า 4 % ในการวิเคราะห์ตัวอย่างน้ำนมตัวอย่างจะถูกเจือจาง 10,000 เท่า ก่อนทำการวิเคราะห์เพื่อ ลดผลจากตัวรบกวน เมื่อวิเคราะห์น้ำนมที่เติมเพนนิซิลินจี ระหว่างความเข้มข้น 1.0×10^{-9} โมลาร์ และ 1.0×10^{-7} โมลาร์ ได้เปอร์เซ็นต์ได้กลับคืนอยู่ในช่วงระหว่าง 64 และ 101 เปอร์เซ็นต์

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ABSTRACT

A flow injection impedimetric immunosensor to determine penicillin G in milk samples was developed. Anti-Penicillin G was immobilized on self-assembled thioctic acid monolayer via covalently coupling on gold working electrode. The interaction between penicillin G and anti-Penicillin G on electrode surface causes the impedance to increase. Real time monitoring of the impedance was carried out at the optimum frequency, 160 Hz. Under optimum operating conditions, linearity was in the range of 1.0×10^{-13} to 1.0×10^{-8} M with a detection limit of 3.0×10^{-15} M, lower than the MRL (1.2×10^{-8} M), and the analysis time was 10-12 min. The immobilized anti-Penicillin G on self-assemble thioctic acid monolayer gold electrode was stable and after regeneration good reproducibility of the signal could be obtained up to 45 times with a relative standard deviation (RSD) lower than 4 %. Real milk samples were diluted 10,000 times before being analyzed to reduce the matrix effect. Analysis of spiked real samples between 1.0×10^{-9} M and 1.0×10^{-7} M provided recoveries in the range of 64 and 101 %.

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My parents, my brothers and my sister for their helps, loves and attentions throughout my life.

Supaporn Dawan

THE RELEVANCE OF THE RESEARCH WORK TO THAILAND

The purpose of this Master of Science thesis in Analytical Chemistry is to determine penicillin G in milk by impedimetric immunosensor. Comparing to the conventional method, this technique is highly selective and sensitive, can detect analyte with accuracy, using short analysis time. The detection limit of the system is very low. This technique can be applied for the determination of antibiotic in food. Organizations that can use the outcome of this work include

- Ministry of Public Health
- Ministry of Environment and Natural Resource
- Ministry of Education

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CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Benzylpenicillin or penicillin G is a beta-lactam antibiotic frequently used in verinary practice for treatment of ovine mastitis and microbial infections (Boatto *et al.*, 1998; Gustavsson *et al.*, 2004). It is highly effective in acute and chronic gonorrhea, infections due to meningococci, pneumococci and β-hemolytic and anaerobic streptococci (Berkow and Talbott, 1977) and is often used in prevention and treatment of bacteria infection by intramuscular injection. This may lead to the presence of penicillin residues in food of animal origin (milk, meat). A very modest quantity of this compound in milk might be responsible for allergic reaction in human. Benzylpenicillin residues may also be responsible for the development of resistant strains of bacteria (Popelka *et al.*, 2004).

To avoid health hazards due to residues in milk or edible tissues obtained from medicated animal, withdrawal times and Maximum Residue Limit (MRL) have been established by the European Union (EU). EU Regulation 2377/90 sets the MRL for benzylpenicillin in milk at 1.2×10^{-8} M or 4 ppb. In addition, food producers and industries carry out self-monitoring programmes to fulfill requirements for export and consumer concerns regarding the safety of food. For production control and quality control of benzylpenicillin, liquid chromatographic (LC) and microbiological method are recommended (Leszczyńska *et al.*, 1998).

The use of liquid chromatography for quantitative analysis of benzylpenicillin is specific, however, its use has been limited by the sensitivity of the detector, which may not fulfill the requirements to monitor this drug far below MRL (Msagati and Nindi, 2007). Furthermore, liquid chromatography is time-consuming, need sample preparation and expensive (Gaudin *et al.*, 2001). The other method, biological, is based on the diffusion of antibiotic in agar inoculated with sensitive microorganisms. The effect of antibiotic is revealed by the production of zones of

inhibitor, the biological method is also time-consuming since it needs incubation time and also non specific (Leszyńska *et al.*, 1998). There are commercial test kits that have been developed to screen antibiotic residues in milk, but these techniques lack specificity and it is rather time consuming, i.e. 30-180 min (Weimer, 1992).

To achieve the required high selectivity, sensitivity and fast response, an amalgamation of signal transducer and biocomponents offers potential alternatively. Biosensors based on enzyme have been used to determine penicillin and benzylpenicillin. Enzyme penicillinase catalyses the hydrolysis of beta-lactam to penicilloic acid, the pH changes could be detected by potentiometric transducer (Eppelsheim *et al.*, 1995; Leszyńska *et al.*, 1998). Amperometric technique has also been applied to detect the change in pH. This was done by using pH-sensitive redox-active probe molecules where the measured current depended on pH (Stred'ansky *et al.*, 2000). Despite the high specificity and selectivity achieved by enzyme biosensors their concentration ranges and LODs were still above the MRL and, therefore, could not be used to analyse penicillin in milk sample (Eppelsheim *et al.*, 1995; Leszyńska *et al.*, 1998; Stred'ansky *et al.*, 2000).

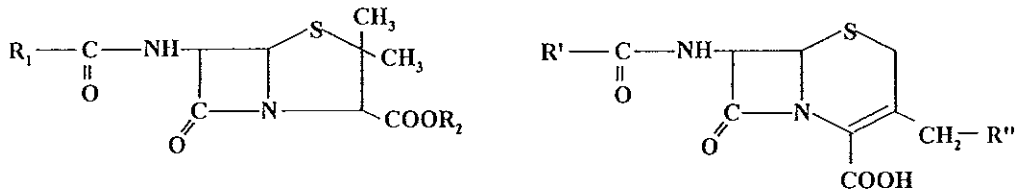
An alternative to enzyme biosensor is affinity biosensor that uses the interaction of antibody-antigen (immunosensor), receptor-lagand or protein-nucleic acid. For the detection of beta-lactam antibiotic in milk, a surface plasmon resonance (SPR) biosensor was developed (Gaudin *et al.*, 2001). The principle of detection was based on the binding of antibody to penicillin at the open beta-lactam ring. The limit of detection of this assay for benzylpenicillin was around MRL (1.2×10^{-8} M). However, since the antibody has higher affinity to the open beta-lactams ring than the closed ring so the sample needed to be pre-treated by using penicillinase enzyme and chemical to obtain open beta-lactam ring before analysis. Although further development is still needed affinity based biosensor seems to provide a better detection limit than the enzyme based and this technique is focused in this work.

To directly investigate the affinity binding reaction impedimetric detection can be employed. This technique is an effective method to probe the interfacial properties of modified electrode and can provide a general method to follow antigen-antibody reaction at modified electrode surface (Katz *et al.*, 2001; Katz and Willner, 2003; Wang *et al.*, 2004). Impedance detection has also been found

to be sensitive, rapid and can detect at low concentration (Dijksma *et al.*, 2001; Ouerghi *et al.*, 2002; Wang *et al.*, 2004; Bart *et al.*, 2005) In this work an impedimetric biosensor based on antibody-antigen binding for benzylpenicillin in milk sample was investigated.

1.2 Use of beta-Lactam antibiotic in daily cows

Beta-lactams, i.e. penicillins and cephalosporins (Figure 1), constitute the group of antibiotic substance most frequently used within veterinary medicine for treatment of infections in dairy cows. The antibacterial effect of beta-lactam substance is due to their inhibition of bacterial cell wall synthesis. The cell wall in Gram-positive bacteria consists largely of peptidoglycans, a network of N-acetylmuramyl and N-acetylglucosaminyl residues that are cross-linked by short peptides. The beta-lactam antibiotics inhibit the transpeptidases that perform the cross-linking of the peptides (Park and Strominger, 1957; Frère and Joris, 1985) and the resulting structural weakness of the cell wall is followed by activation of autolytic enzymes, causing lysis of the beta-lactam (Miller, 2002). Since eukaryotic cell do not have similar cell wall structure, the beta-lactams are not toxic for humans and other eukaryotic species (Greko and Sternberg, 1996).



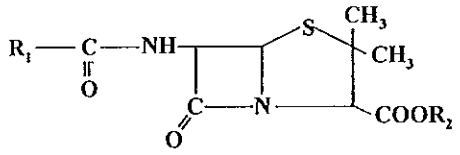
Penicillins **Cephalosporins**

Figure 1 Structure of penicillins and cephalosporins (Gustavsson *et al.*, 2004).

Some bacteria strains have developed resistance mechanisms against the most common penicillins, which consequently cannot be used in therapy against these bacteria. Chemical modifications of the natural penicillin structure have resulted in beta-lactam with board specificity and improved efficiency (Greko and Sternberg,

1996; Miller, 2002), even though, penicillins is still the most commonly used in livestock farming and bovine milk.

Penicillin is not a single compound but a group of closely related compounds, all with the same basic ring-like structure (a beta-lactam) (Figure 2). The nature of the acyl group (R_1 in the Figure 2) confers specific properties on different types of penicillin.



Beta-Lactam (Basic structure)

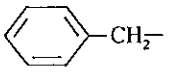
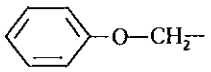
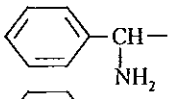
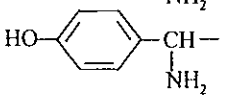
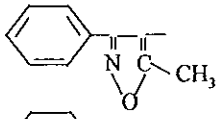
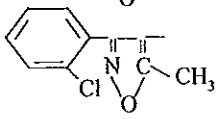
Compound	R_1	R_2
Penicillin G		H
Penicillin V		H
Ampicillin		H
Amoxicillin		H
Oxacillin		H
Cloxacillin		H

Figure 2 General structure of penicillins (Modified from Gaudin *et al.*, 2001).

Penicillins can be classified into three groups

(a) Natural penicillins include penicillin G (PENG) and penicillin V (PENV).

(b) Penicillinase-resistant penicillins, these include methicillin (METH), nafcillin (NAFCI), oxacillin (OXA), cloxacillin (CLOX), dicloxacillin (DICLOX) and mecillinam (MECI).

(c) The broad spectrum penicillins include ampicillin (AMPI), amoxicillin (AMOX) and hetacillin (HETA) (Benito-Peña *et al.*, 2006).

All penicillins are qualitatively similar in their antimicrobial spectrum, but differ significantly in their degree of effectiveness against specific species and strains of bacteria. Although each of the semi-synthetic penicillin has advantages, penicillin G remains the preferred agent for majority of penicillin-susceptible infection. Other penicillins frequently are not as active as penicillin G against susceptible organisms, including non-penicillinase-producing staphylococci (Berkow and Talbott, 1977). Therefore, penicillin G is still widely used to treat animal bacterial infections. Penicillin has also been added in animal feed as a growth promoter.

Because of its low toxicity and cost, and the increasing resistance of microorganisms to penicillin over the year, users started to increase the dosage. Such use of penicillin has contributed to residues in food (mik, meat) (Dubreuil *et al.*, 2001), which presents possible health hazards to consumers. Although side effects of penicillin which are true toxic reactions seldom occur, however, allergic reactions are not uncommon. Hypersensitivity response includes;

(i) Skin rashes (urticaria; erythematous, macular, popular, morbilliform, scarlatiniform, or purpuric rashes; bullous eruptions; exfoliative dermatitis) appearing 7 to 10 days after therapy begins, and rarely, persisting or recurring for weeks after therapy is stopped.

(ii) Serum sickness.

(iii) Anaphylactic shock with sudden death after oral or parenteral administration (Berkow and Talbott, 1977).

Because of these possible effects of penicillin several institutes such as European Union (EU), Positive List System in Food Sanitation Law: Japan, are limiting the maximum residues (MRLs) of penicillin in food (Table 1).

Table 1 Maximum Residues Limits (MRLs) of beta-lactams in food (EEC, 1990; ACFS, 2006).

Beta-lactams	MRL (ppb)	
	EU	Japan
Penicillin G	4	4
Amoxilcillin	4	8
Ampicillin	4	20
Cloxacillin	30	50
Oxacillin	30	300
Cefalexin	100	100
Cephapirin	60	30
Ceftiofur	100	100

Penicillin, especially benzylpenicillin is usually given by intramuscular injection at dose 10,000 IU/body weigh and prolonged milk residues have been report up to 3 days in cow (LABORATORIOS HIPRA, S.A., Spain). Therefore, there will be some benzylpenicillin residue if milking is done within the 3 days. Case study reported by Kaneene and Ahl (1987) indicated that when penicillin residues were found in bulk tank milk 30% were related to insufficient knowledge about withdrawal periods, 23% were due to errors from employees, 14% from poor identification or record, 12% to metritis treatment, 7% to dry cow treatment for mastitis, and 4% for incorrect label reading (Kaneene and Ahl, 1987). That is, even with current knowledge of treatment and withdrawal time benzylpenicillin residues still exist in milk. Therefore, a sensitive and reliable method is needed. Since the MRLs of benzylpenicillin have been set at low concentrations (Table 1), it is necessary to develop the method that can detect at these levels, especially in milk and milk products because they are the basic food, particularly for infants, young children, sick and old persons.

1.3 Conventional Method for determination of penicillin G

There are several methods that are available for the determination of penicillin G residues (Boatto *et al.*, 1998). These include instrumental and biological techniques as well as several test kits.

1.3.1 Liquid Chromatography

High Performance Liquid Chromatography (HPLC), coupled with various detectors, is the most widely used instrumentation method for quantitative analysis of penicillin G. The analysis was performed with a C18 column (Tarbin *et al.*, 1995; Boatto *et al.*, 1998; Popelka *et al.*, 2003). The detectors that have been used include Ultraviolet (UV) or Ultraviolet-diode array detection (UV-DAD) (Tarbin *et al.*, 1995; Lihl *et al.*, 1996; Boatto *et al.*, 1998; Furusawa, 2000; Marchetti *et al.*, 2001; Popelka *et al.*, 2003; Loksuwan *et al.*, 2005) and mass spectrometry (MS) (Riediker *et al.*, 2001; Ghidini *et al.*, 2002; Riediker *et al.*, 2004). Among these, liquid chromatography coupled with UV or UV-DAD detector is the usual technique use to detect penicillin G because this detector is simple to use, fast and precise. However, it needs several sample preparation steps.

The most widely used method for preparation of milk sample is liquid-liquid extraction (LLE). In this technique milk sample was deproteinated with reagents (such as acetonitrile, methanol) and cleaned up through a C18 cartridge (Boatto *et al.*, 1998; Takeba *et al.*, 1998) followed by UV or UV-DAD absorbance determination at 220 nm. The limit of detection of penicillin G of this method was 2.6 x 10^{-8} M, higher than the MRL (Boatto *et al.*, 1998; Msagati *et al.*, 2007).

To improve the sensitivity and detection limit derivatization has been investigated. In this method, milk sample was deproteinated followed by cleaned up step on a C18 solid phase extraction column, derivatized with 1,2,4-triazole and mercury (II) chloride reagent (Figure 3), and detected by UV or UV-DAD at 320 nm (Tarbin *et al.*, 1995; Furusawa *et al.*, 2000; Marchetti *et al.*, 2001; Brito and Junqueira, 2006). Penicillin G could be detected at 9.0 x 10^{-9} M, below the MRL (Brito and Junqueira, 2006). However, this technique requires toxic reagent such as mercury in the derivatization steps. To overcome this problem liquid chromatography coupled with mass spectrometry (LC-MS) has been used as a highly sensitive and

selective tool. It has been applied to detect penicillin G in milk with a detection limit of 6.0 x 10^{-9}M (Riediker *et al.*, 2001).

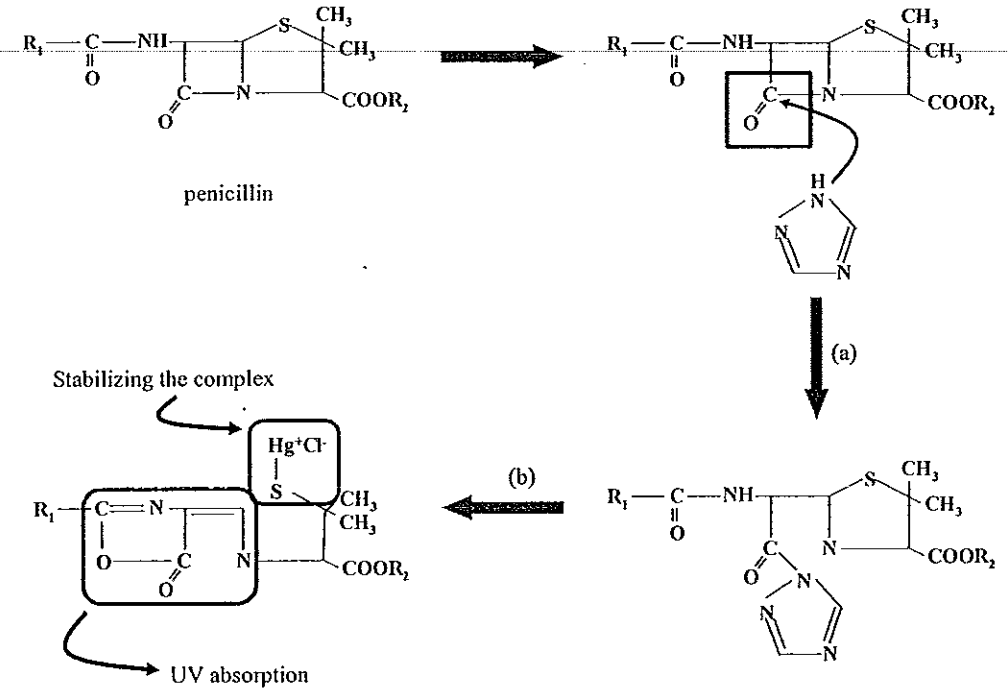


Figure 3 Mechanism of the derivatization of penicillin (a) opening of the lactam ring catalyzed by 1,2,4-triazole, (b) formation of the N-acetyl-penicillinic acid – Hg(II)$^{-}$ mercaptide (Marchetti *et al.*, 2001).

Although, LC techniques could be used to detected penicillin G with relatively low detection limit, they are expensive, require large amount of high purity organic solvents, long system equilibration, special sample preparation and time-consuming (Gaudin *et al.*, 2001; Nozal *et al.*, 2004). In recent years, the use of capillary electrophoresis (CE) for determination of pharmaceuticals and drugs has gained considerable importance and it has been extensively discussed in some papers (Michalska *et al.*, 2004; Pajchel *et al.*, 2005).

1.3.2 Capillary electrophoresis

CE is a modern and advantageous tool in the chemical analysis of pharmaceutical (Michalska *et al.*, 2004; Pajchel *et al.*, 2005) because of its good selectivity (Puig *et al.*, 2006). This method provides adequate separation and simplicity for method of quality control. It is performed in a narrow-bore fused silica capillary which is filled with buffer. A schematic diagram of a generic capillary electrophoresis system is shown in Figure 4. The sample is loaded into the capillary by replacing the anode reservoir with a sample reservoir and applying an external pressure. The electric field is then applied and the separation performed. A detector such as UV-visible is placed at the opposite end of the capillary (Nishi and Terabe, 1995; Lagane *et al.*, 2000; Pajchel *et al.*, 2004; Puig *et al.*, 2006).

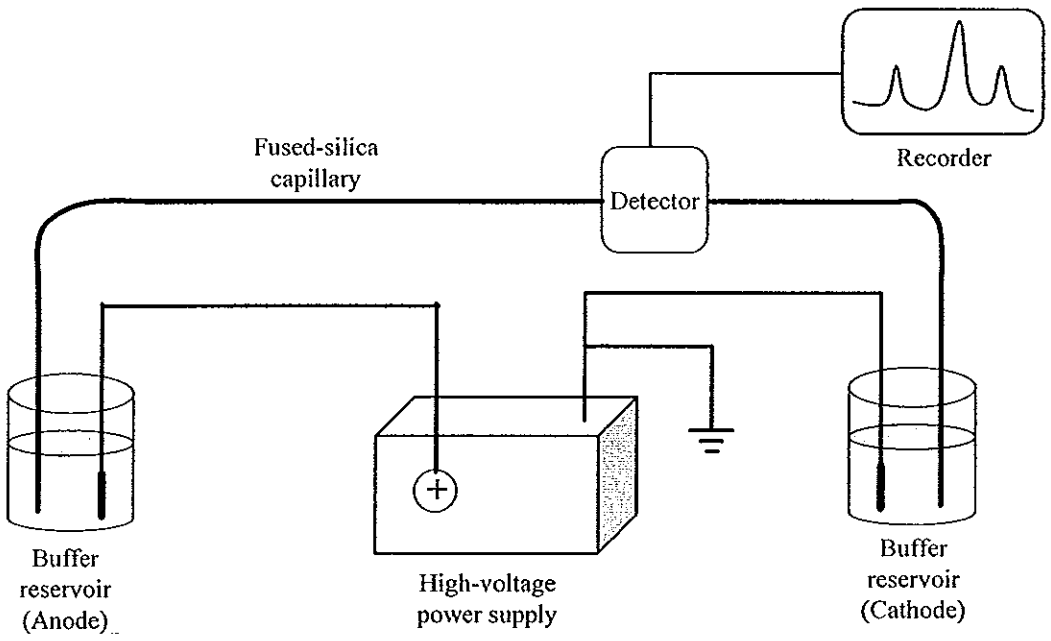


Figure 4 Instrumental set-up of a capillary electrophoresis system (Lagane *et al.*, 2000).

Capillary electrophoresis (CE) has many advantages in comparison with HPLC. The purity of organic solvents in the running buffer needs not be as high as in HPLC, short running time for the separation of the analytes, low operation cost

and high separation efficiency (Li *et al.*, 1998; Nozal *et al.*, 2004) and has proven to be a significant and versatile technique for analysis of penicillin (Sciacchitano *et al.*, 1994; Emaldi *et al.*, 1995).

Most CE methods use to analyze antibiotic compounds involving micellar electrokinetic chromatography (MEKC) or micellar electrokinetic capillary chromatography (MECC) (Emaldi *et al.*, 1995; Altria, 1999; Nozal *et al.*, 2004; Puig *et al.*, 2006). Typically the method is performed using high pH buffer containing sodium dodecyl sulphate (SDS). The SDS is usually present in the range of 20-100 mM where the SDS surfactant molecules aggregate to form negatively charged micelles. Neutral solutes partition into the micelle depending upon their hydrophobicity (solubility). The negatively charged SDS micelles attempt to migrate against the electroendosmotic flow (EOF) and therefore hydrophobic solutes are retained by the micelle and are detected late (Altria, 1999).

MEKE has been used for separation of penicillin compounds; the analysis was performed with a voltage of 10-30 kV. The samples were hydrostatically introduced into the capillary and response of antibiotic such as amoxicillin, ampicillin and penicillin G were recorded at 210 nm by a diode array detector (Nozal *et al.*, 2004) and UV spectrophotometric detector (Pajchel *et al.*, 2004). Although this technique has some advantages over HPLC but it still gave high detection limit, 6.0 x 10^{-7} M for penicillin (Nozal *et al.*, 2004) and 1.1 x 10^{-5} M for benzylpenicillin (Pajchel *et al.*, 2004), higher than the MRL.

1.3.3 Microbiological methods

American Organization of Analytical Chemists (AOAC) recommends several methods for determining beta-lactam antibiotics in milk and fluid milk products. Method 982.15 is based on specific, irreversible affinity of beta-lactam antibiotics for certain enzyme sites on cell wall of microorganisms. ^{14}C-labeled penicillin and *Bacillus stearothermophilus* are added to milk sample. Antibiotic in the sample competes with ^{14}C-penicillin for binding sites. Amount of bound carbon-14 is counted and compared with control to determine the presence of bete-lactams antibiotic, this technique is applicable to $\geq$ 6 ppb of penicillin G or beta-lactam

equivalent. The analysis time for this method is more than 30 min (AOAC international, 1996).

Method 982.18 is based on rapid growth rate and acid production of *Bacillus stearothermophilus* var. *calidolactis*. Acid changes color of bromcresol purple to yellow in absence of beta-lactam inhibitors. In presence of inhibitors, purple color remains. This method is applicable for detecting and confirming levels of beta-lactam residues ≥ 3 ppb in processed fluid milk products and raw milk with analysis time of about 3 h. Test may be performed by using ampule or multitest kits (Delvotest®) (AOAC international, 1996).

Method 988.08 is based on competitive binding reaction between labeled antibiotic and antibiotic in the sample to added microbial cells. The drug was labeled with isotope of ^{14}C and ^{3}H, e.g. penicillin labeled with ^{14}C (Penicillin^{14}C) and tetracycline labeled with ^{3}H (tetracycline^{3}H). The labeled drug and known amount of microbial cells were added into the sample. Antibiotic in sample competed with labeled drug for the receptor sites on added cells. Binding is measured by scintillation counter and compared with zero standard milk. The greater the amounts of antibiotic present in the sample, the lower the counts. This method does not detect metabolites, only active drugs. The validate levels (ppb) are penicillin G, 4.8; cephapirin, 5; cloxacillin, 100; chlorotetracycline, 2000; oxytetracycline, 2000; tetracycline, 2000; erythromycin, 200; lincomycin, 400; chlindamycin, 400; sulfamethazine, 75; sulfamethoxazole, 50; sulfasoxazole, 50; streptomycin, 1000; novobiocin, 50 and chloramphenicol, 800. The analysis time for this method is more than 30 min (AOAC international, 1996).

Microbiological tests are commonly used as screening methods. However, these tests are time-consuming, lack sensitivity for diverse groups of antibiotic and do not allow substance identification (Leszczyńska *et al.*, 1998; Knecht *et al.*, 2004).

1.3.4 Commercial Test kit for beta-lactam in milk

Several commercial test kits are available for screening raw milk. **Charm II®** beta-lactam test is similar to AOAC method 988.08. It was used to semi-quantitatively determine penicillin G, the limit of detection was below 2 ppb for

penicillin G (Grunwald and Petz, 2003; Popelka *et al.*, 2004). This test is relatively expensive, 350 baht per test and it takes 3 h (Sangvits Science,Thailand).

Delvotest SP or **Delvotest®** is based on method 982.12 of AOAC, in cow milk this test is sensitive at 3 ppb and 8 ppb of penicillin G and cephapirin, respectively. It takes 2.5 – 3 h, similar to Charm II®. (Zeng *et al.*, 1996; Popelka *et al.*, 2004) but is cheaper, 100 baht ($2.66) per test (Weimer, 1992).

Penzyme® test is based on enzyme assay and used as a screening method. Carboxypeptidase causes a color change in the content of the test vial in the absence of antibiotics and an orange/pink color appears. With the presence of sufficient beta-lactam antibiotics in milk, the enzyme forms a stable and inactive complex and the yellow color of the content of the vial remains. The detection levels of the Penzyme® test are 5 ppb and 8 ppb of penicillin and cephapirin in cow milk, respectively and results are obtained in 20 min (Zeng *et al.*, 1996) at 90 baht ($2.28) per test (Weimer, 1992).

These commercial test kits can only be used as screening method for antibiotic in milk, are time-consuming with relatively high cost (Chan *et al.*, 2004; Knecht *et al.*, 2004). Therefore, some alternative methods are still needed.

1.4 Biosensor

Biosensor is a device combining a biological recognition element and a suitable transducer. The biological recognition element of a biosensor interacts selectively with the target analytes, assuring the selectivity of the sensors (Figure 5). These elements can be classified into two main classes: catalytic (e.g. enzymes, microorganisms, tissue materials) and affinity (e.g. antibodies, nucleic acids, lectins) (Figure 6) (Gaudin *et al.*, 2001; Castillo *et al.*, 2004).

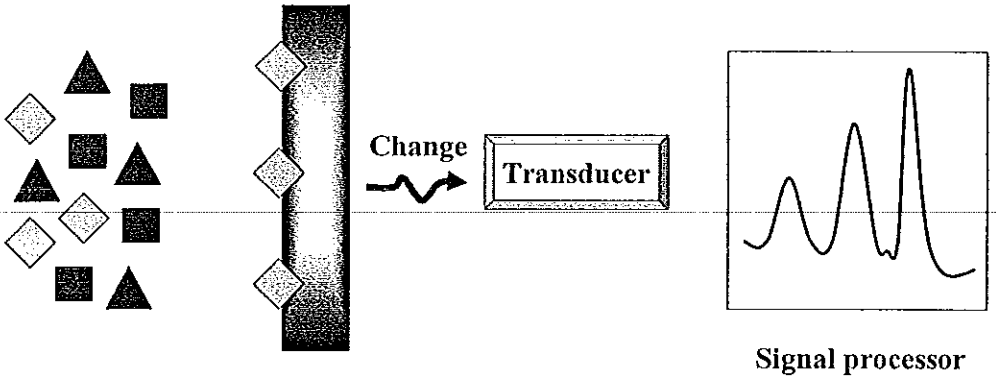


Figure 5 Schematic illustration of the components of a biosensor.

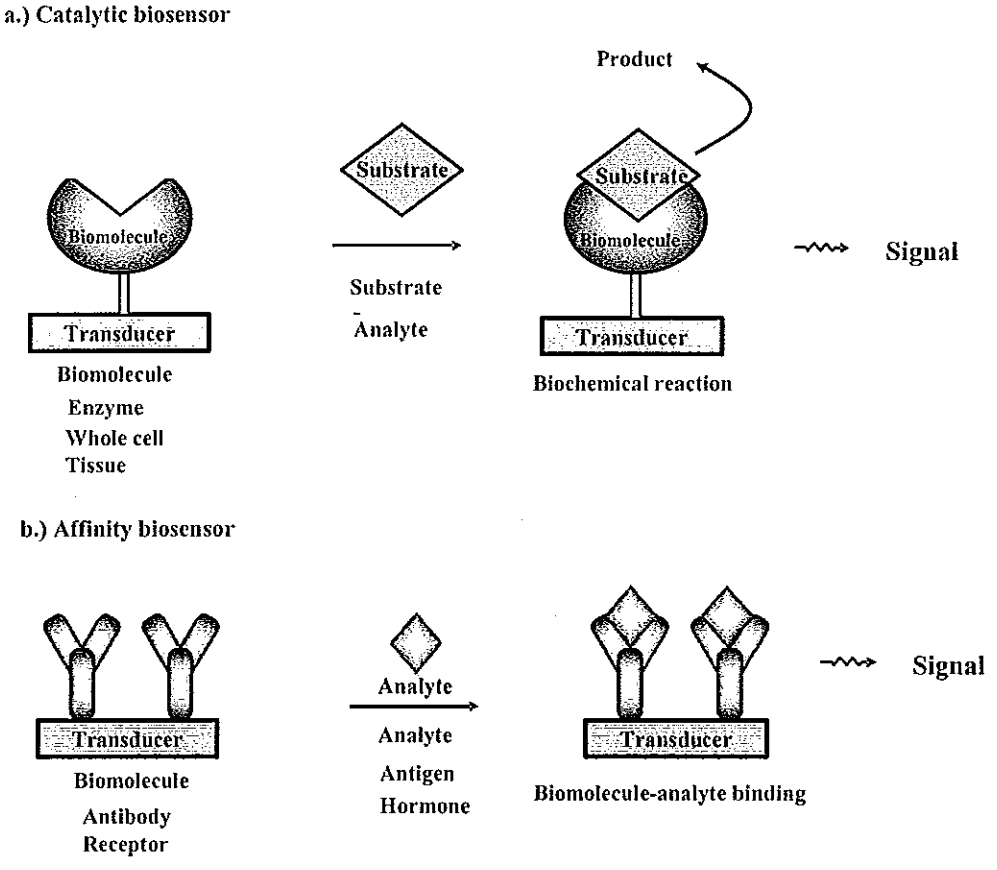


Figure 6 Major biosensor types.

Biosensors are versatile tools applied more and more in different field, such as medicine, food quality and safety control, and environmental pollutant monitoring (Castillo *et al.*, 2004). Biosensors offer some advantages over classical analytical techniques in term of selectivity, sensitivity and reduce cost of analysis (Collings and Caruso, 1997), potential for miniaturization, facility of automation and simple and portable equipment construction for a fast analysis and monitoring in platforms of raw material reception, quality control laboratories or some stage during the food processing (Luong *et al.*, 1997).

The developments of biosensors could be employed in foods, especially applied to determination of the composition, degree of contamination of raw materials and processed foods, and for the on line control of the fermentation process. In the food industry attractive areas for biosensor application are the detection of pathogens such as bacteria (Ye *et al.*, 1997; DeMarco *et al.*, 1999; Hamid *et al.*, 1999) toxin (Carter *et al.*, 1997; Rasooly and Rasoly, 1999; Rasooly, 2001), pesticide in milk (Medyantseva *et al.*, 1998), pesticide in fruit and vegetable juices (Palchetti *et al.*, 1997; Pita *et al.*, 1997; Nunes *et al.*, 1999), herbicide in food (Yulaev *et al.*, 2001), herbicide in vegetables (Starodub *et al.*, 2000), herbicide in drinking water (Steegborn and Skládal, 1997) and antibiotic in food (Sternesjö *et al.*, 1995; Mellgren and Sternesjö, 1998; Bergstrom *et al.*, 1999; Baxter *et al.*, 2001; Gaudin and Maris, 2001; Gaudin *et al.*, 2001; Haasnoot and Verheijen, 2001; Traynor *et al.*, 2003).

For antibiotics biosensors have been investigated to determine streptomycin (Barter *et al.*, 2001), sulphamethazine (Bergstrom *et al.*, 1999), gentamicin (Haasnoot and Verheijen, 2001), ciprofloxacin and enroloxacin (Mellgran *et al.*, 1998), sulfamethazine (Sternesjö *et al.*, 1995), beta-agonist (Traynor *et al.*, 2003; Gustavsson, 2004), chloramphenicol (Gaudin and Maris, 2001; Park *et al.*, 2004) and penicillin (Eppelsheim *et al.*, 1995; Poghossian *et al.*, 2000; Stred'anský *et al.*, 2000; Gaudin *et al.*, 2001; Poghssian *et al.*, 2001; Gustavsson, 2003).

In the case of penicillin, the biological sensing elements that have been employed were enzymes (Eppelsheim *et al.*, 1995; Leszcyńska *et al.*, 1998; Poahossian *et al.*, 2000; Stred'anský *et al.*, 2000; Poghossian *et al.*, 2001; Gustavsson *et al.*, 2002; Gustavsson *et al.*, 2004) and antibodies (Gaudin *et al.*, 2001), and the transducers were either optical (Sternesjö *et al.*, 1995; Gusavsson, 2003; Traynor *et*

al., 2003; Johansson, 2004) or electrochemical (Leszcyńska *et al.*, 1998; Poghssian *et al.*, 2001).

1.4.1 Enzymatic biosensor for penicillin

Enzymes are frequently used as the biological component in biosensors. Enzyme penicillinase has been used to determine penicillin where the enzyme converts penicillin in aqueous solutions to penicilloic acid which resulted in a pH change (Figure 7). The detection often employ potentiometric detection principle to directly detect pH where the intensity of the signal increases with the concentration of penicillin. pH sensing has been carried out using pH glass electrode (Eppelsheim *et al.*, 1995), ion-selective membrane or film electrodes (Eppelsheim *et al.*, 1995; Leszczyńska *et al.*, 1998) and ion-selective field effect transistors (ISFET) (Poghossian *et al.*, 2001).

The incorporation of penicillinase enzyme to the sensing part was done by several methods. Penicillinase has been immobilized by covalent binding directly to the membrane surface this technique has a linear range of $1.6 \times 10^{-3} - 2.2 \times 10^{-3}$ M with daily usage it can be used about 2 months (Leszczyńska *et al.*, 1998). The immobilization onto transducer has also been done by aqueous dispersion polymer. In this case the performance was not as good as the previous method. The linear range was between 4.0×10^{-2} and 1.0×10^{-1} M, LOD 1.5×10^{-2} M, and the electrode could be used for 2 weeks (Eppelsheim *et al.*, 1995). For a penicillin-sensitive field-effect transistor (PenFET) the enzyme was adsorptively immobilized. In this system the linear range was $5.0 \times 10^{-5} - 1.0 \times 10^{-3}$ M, LOD 5.0×10^{-6} M and had a very long lifetime of more than 1 year (372^{nd} day). During this period, the sensor was periodically checked for more than 300 measurements (Poghossian *et al.*, 2001). Although the last technique has a very long lifetime and the lowest LOD it is still not suitable to be used to determine penicillin in milk sample because its LOD is higher than the set MRL (1.2×10^{-8} M).

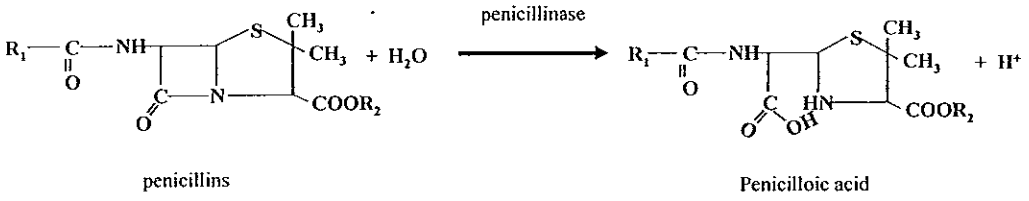


Figure 7 The penicillinase enzyme hydrolysis of penicillin (Modified from Lapierre *et al.*, 1999; Gaudin *et al.*, 2001).

Besides potentiometric, amperometric technique has also been applied to detect penicillin by enzymatic biosensor. The enzyme was prepared by placing penicillinase on the platinum electrode surface (working electrode) which was then covered by dialysis membrane, the stability was about 1 month. The amperometric pH-sensing was based on pH-sensitive redox-active probe molecules where dissolved hematein was used to determine penicillin. Hematein is a natural dye used in the selective staining of biological material. It is a stable water-soluble electroactive compound also employed as a pH indicator, turning from yellow at active pH to pinkish pH values at basic pH. The working potential of 0 mV was applied for amperometric measurements. The dependence of current on pH was determined using a platinum electrode immersed in a 0.5 mM hematein solution. Changing the pH of the buffer solution, from 3.9 – 9.8, the resulting current response was found to be reversible without affecting the magnitude of different current (ΔI) and the response time at steady state at either pH extreme. The biosensors exhibited low detection limit, from 2-10 µM (Stred'anký *et al.*, 2000).

Recently another biosensor was developed to detected penicillin G in milk based on enzymatic activity of carboxypeptidase (R39) to convert a 3-peptide into a 2-peptide (Figure 8). This reaction is inhibited by beta-lactams. Antibodies were used to measure either the amount of formed enzymatic product or the amount of remaining enzymatic substrate. Both assays detected different beta-lactam at below MRL, and the detection limit of penicillin G was 3.6×10^{-9} M and 4.5×10^{-9} M for 2-peptide and 3-peptide assay, respectively (Gustavasson *et al.*, 2002; Gustavasson *et al.*, 2004). However, this technique was affected by the high variation due to the non-

specific binding between different milk samples that made it impossible to use this assay to screen field samples. The assay might probably have been improved by using a lower fraction of milk in the injected sample mixture (Gustavsson, 2003).

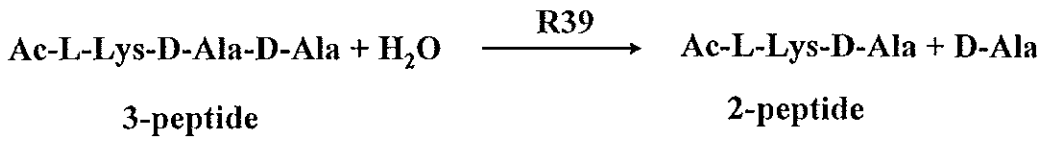


Figure 8 Enzyme reaction of hydrolysis of a 3-peptide into a 2-peptide catalyzed by R39 (Gustavsson, 2003).

1.4.2 Affinity biosensor for penicillin

Affinity biosensors exploit selective binding of certain biomolecules toward specific target species. The biomolecular recognition process is governed primarily by the shape and size of the receptor pocket and the ligand of interest (the analyte). The high specificity and affinity of these biochemical binding reactions lead to highly selective and sensitive sensing devices (Wang, 2000). Affinity biosensors can be classified according to the biological sensing element into DNA biosensors, receptor biosensors and immunosensors (antigen-antibody).

This review will focus on affinity biosensor based on antigen-antibody binding, i.e. immuonosensor. Immunosensor can be divided into two categories; labeled and label-free (Ghindilis *et al.*, 1998; Luppa *et al.*, 2001; Orazio, 2003). The labeled immunosensors use the same principle as labeled immunoassay. To be able to detect the interaction, one of the immunoagents has to be labeled by a signal-generation component. A labeled component is used to generate a signal which enables quantification of the amount of bound antigen (Ag) relative to unbound Ag. Among the most valuable labels are enzymes such as horseradish peroxidase, glucose oxidase (Ghindilis *et al.*, 1991; Skladal and Kalab, 1995; Luppa *et al.*, 2001; Knecht *et al.*, 2004) and fluorescent molecules (Benito-Peña *et al.*, 2005).

A labeled immunosensor to detect antibiotics in milk has been investigated (Knecht *et al.*, 2004). Microscope glass slide modified with (3-glycidyloxypropyl) trimethoxysilane was used for the preparation of hapten

microarrays. Protein conjugates of the haptens were immobilized as spots on disposable chips, which were processed in a flow cell. Monoclonal antibodies against penicillin G, cloxacillin, cephapirin, and tylosin allowed the simultaneous detection of the respective analytes. Antibody binding was detected by a second antibody labeled with horseradish peroxidase generating enhance chemiluminescence, which was recorded with a sensitive CCD camera. The detection limits ranged from 0.12 (cephapirin) to 32 ppb (neomycin). Penicillin G could be detected at the MRL and the detection limits for all other analytes were far below their respective MRLs. However, the assays are more suitable for the screening because of their narrow working ranges. The MRL concentration was outside the working range for most analytes, this providing a clear positive readout but limiting quantification (Knecht *et al.*, 2004).

In another immunosensor, an immunogen was prepared by coupling the common structure of the penicillanic beta-lactam antibiotics. Polyclonal antibodies raised in rabbits after immunization with this conjugate have been applied for the development of a competitive fluoroimmunoassay (FIA). Penicillin was labeled with a novel fluorescent ([2S, 5R, 6R]-3, 3-dimethyl-7-oxo-6-[(pyren-1ylacetyl) amino-4-thia-1-azabicyclo [3.2.0] heptane-2-carboxilic acid) (abbreviated-PAAP). The measuring protocol was based on the principle of a competitive direct fluoroimmunoassay. The sample (with penicillin G) was mixed with a constant amount of labeled antigen (PAAP) and antibody. The unlabeled analyte (Ag: penicillin G) compete with a labeled compound (Ag*: PAAP) for a limited number of antibody. The mixture was then injected into an immunoreactor containing immunosorbent that will bind with the Fc part of the antibody. The reactor was then washing to remove the unbound components. Upon desorption from the immunosupport, the emission signal generated by the PAAP-Ab complexes is related to the antibiotic concentration in the sample. The detection limit was 2.4 ppb for penicillin G (Benito-Peña *et al.*, 2005). Although the LOD is low the procedure requires several steps.

Another category of immonosensor is label-free. These sensors are based on the detection of physical changes during antigen-antibody (Ag-Ab) complex formation. Generally the surface of a transducer is modified by either antigen or antibody. Antigen-antibody complex formation then leads to a change in surface

properties (Luppa *et al.*, 2001). This technique has been used to detect several antibiotics, such as sulfamathazine (Sternesjö *et al.*, 1995), streptomycin (Baxter *et al.*, 2001), chloramphenicol (Gaudin *et al.*, 2001) and penicillin (Gaudin *et al.*, 2001) using surface plasmon resonance (SPR). The testing of these antibiotics followed the same procedure. The antigen was covalently immobilized on a sensor chip. Spiked samples with known concentrations of antigen were prepared in buffer and skim milk for construction of standard curve. Antibodies were added to the sample some of which would bind to the antigen in the sample. The solution was then injected into biosensor system where the free antibodies would then bound with the immobilized antigen on the sensor surface and was detected by SPR. High response would be obtained when the amount of free antibodies was high, i.e. when the analyte (antigen) in the sample was low and vice versa. After each measurement the surface was regenerated to remove the bound antibody from the sensor surface by NaOH and HCl and then reused. The detection limits of the antibiotics were below MRLs. For penicillins a pre-treatment step was required, using penicillinase or chemical pre-treatment to open the beta-lactam ring since the antibodies used in these tests had a much higher affinity for open beta-lactams ring than for closed ring, LOD for penicillin G was 4 ppb, the same as the MRL (Gaudin *et al.*, 2001).

In another approach antibodies for gentamicin (Haasnoot and Verheijen, 2001) and chloramphenicol (Park *et al.*, 2004) were immobilized on the sensor chips. The binding of the antigen (analyte) to the antibody was detected by SPR for gentamicin and quartz crystal microbalance (QCM) for chloramphenicol. The detection limit for gentamicin (10 ppb) is below MRL (100 ppb) but for chloramphenicol (1.0×10^{-5} M) was above MRL (7.8×10^{-8} M).

1.4.3 Impedimetric immunosensors

Label-free immunosensors use numerous transduction techniques, some of which have been mentioned in the previous section such as quartz crystal microbalance (QCM) (Park *et al.*, 2004) and surface plasmon resonance (SPR) (Vikinge *et al.*, 1998; Harris *et al.*, 1999). Electrochemical technique is also a possibility (Willner *et al.*, 1994). Among the various transduction techniques, impedance spectroscopy is an effective method to probe the interfacial properties of

modified electrode and often used for understanding chemical transformation and processes associated with the conductive supports (Ren and Pickup, 1997; Janek *et al.*, 1997; Brillas *et al.*, 1997; Bard and Faulkner, 2001; Tang *et al.*., 2004). The electrified interfaces have gradually evolved from repeated modification of model first proposed by Helmholtz (Figure 9(a)).

Impedance is a totally complex resistance encountered when a current flows through a circuit made of resistor, capacitor, or inductors, or any combination of these. Depending on how the electronic components are configured, both the magnitude and the phase shift of an alternating current can be determined. Because an inductive effect is not usually encountered in electrochemistry a simple equivalent circuit as shown in Figure 9(b) can be considered (Park and Yoo, 2003).

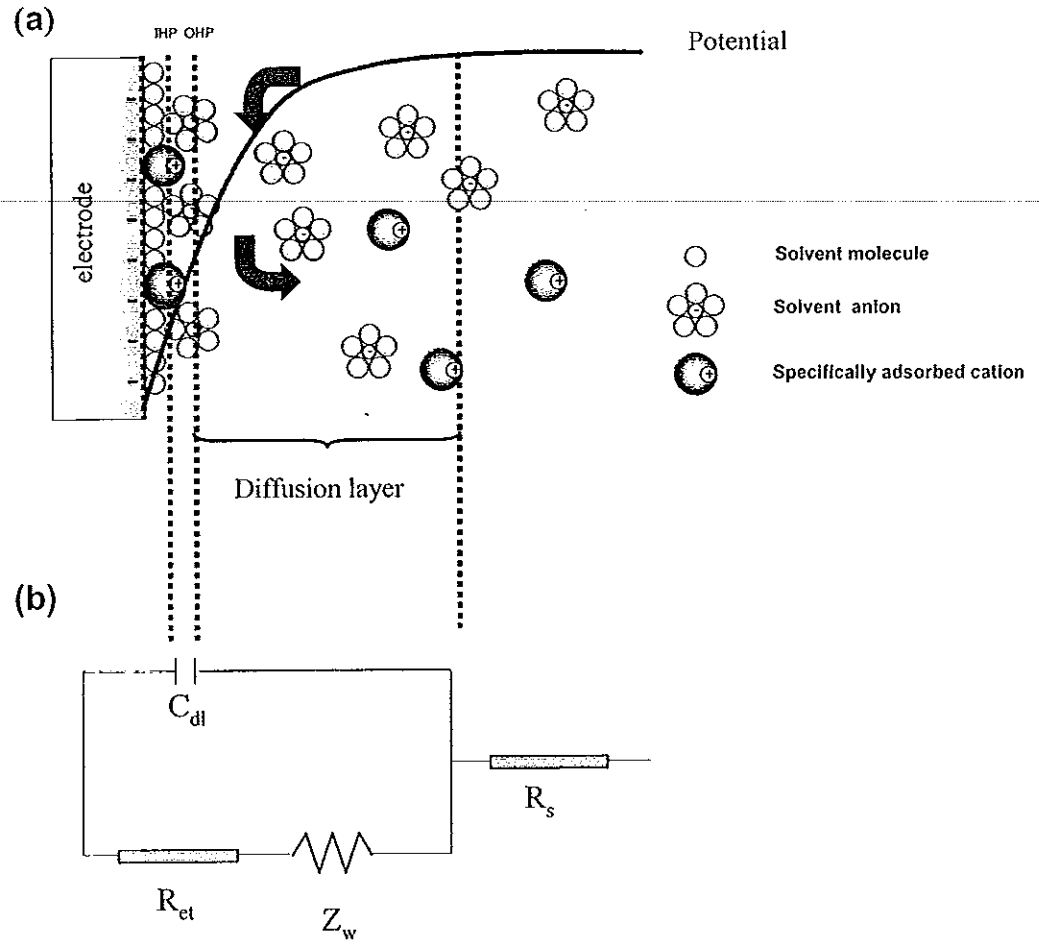


Figure 9 A simple electrified interface, in which the vertical dotted lines in (a) are represented by the electronic components in (b).

(a)The oxidants with a positive charge diffuse toward the negatively charged electrode, accept electrons from the electrode at the interface, become the reductants, and diffuse to the bulk of the solution. The oxidant is also a counterion to the electrode. No specific adsorption is considered at the interface. IHP and OHP are the inner and outer Helmholtz planes, respectively. (b) An equivalent circuit representing each component at the interface and in solution during an electrochemical reaction is shown for comparison with the physical components. C_{dl} is double layer capacitor, R_{et} is electron transfer resistor, Z_w is Warburg resistor and R_s is solution resistor (Modified from Park and Yoo, 2003).

The equivalent circuit (Figure 9(b)) includes the ohmic resistance of the electrolyte solution, R_s, the Warburg impedance, Z_w, resulting from the diffusion of the ions from the bulk electrolyte to the electrode interface, the double layer capacitance, C_{dl} and electron transfer resistance, R_{et}. Since R_s and Z_w represent bulk properties of the electrolyte solution and diffusion features, therefore, they are not affected by chemical transformations occruing at the electrode surface (Katz and Willner, 2003). To make the derivation of the equation and its interpretation straightforward, the Warburg component can be neglected (Park and Yoo, 2003). The other two components in the circuit, C_{dl} and R_{et}, depend on the dielectric and insulating features at the electrode/electrolyte interface, and are controlled by the surface modification of the electrode. In fact, the electron transfer resistance, R_{et}, controls the interfacial support (Alfonta *et al.*, 2001; Katz *et al.*, 2001). The impedance of the interface consists of two part, a real part, Z' and imaginary part, Z" with a complex representation (Equation 1) (Bard and Faukner, 2001; Katz *et al.*, 2001; Katz and Willner, 2003).

$$Z(\omega) = R_s + \frac{R_{et}}{1 + j\omega R_{et} C_{dl}}$$

$$= R_s + \frac{R_{et}}{1 + \omega^2 R_{et}^{\ 2} C_{dl}^{\ 2}} - \frac{j\omega R_{et}^{\ 2} C_{dl}}{1 + \omega^2 R_{et}^{\ 2} C_{dl}^{\ 2}}$$

$$= Z'(\omega) + jZ''(\omega) \qquad (1)$$

A typical shape of a Faradaic impedance spectrum is presented in the form of an impedance complex plane diagram, a Nyquist plot, plot between imaginary part (Z") and real part (Z'). This includes a semicircle region lying on the axis followed by a straight line as shown Figure 10. The semicircle portion (Figure 10 (a)), observed at high frequencies, corresponds to the electron transfer limited process, whereas the linear part of the spectrum (Figure 10 (b)) is characteristic of the lower

frequency range and represents the diffusional limited electron transfer process (Kharitonov *et al.*, 2000; Katz and Willner, 2003).

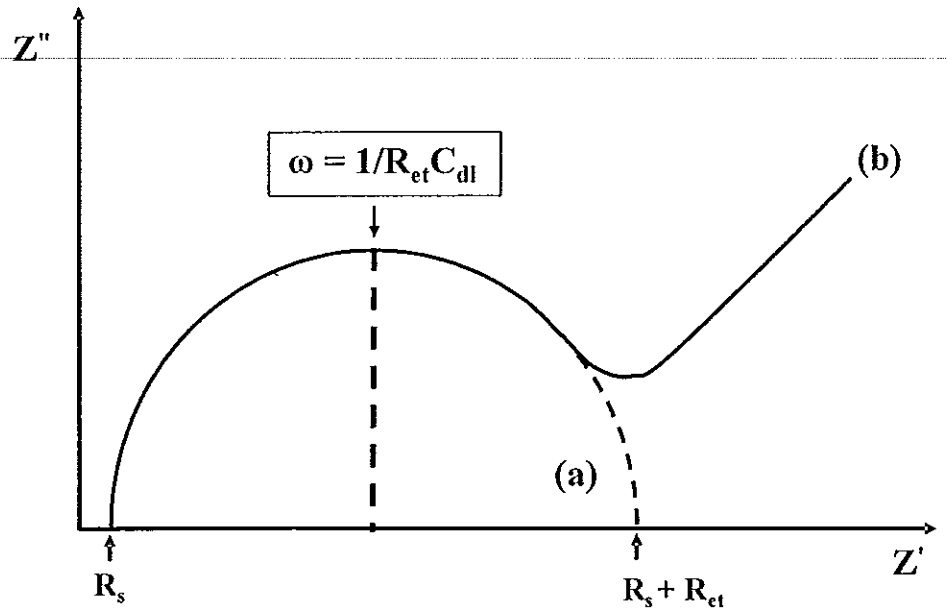


Figure 10 Nyquist plot; $Z^{'}$ = real part of impedance, $Z^{"}$ = imaginary part of impedance, ω = the radial frequency, R_s = solution resistance, R_{et} = electron-transfer resistance and C_{dl} = double layer capacitance (modified from Kharitonov *et al.*, 2000; Katz and Willner, 2003).

Extraction of the system characteristic requires interpreting the Nyquist plot (Figure 10) according to equation 1. At high frequencies the frequency-dependent term of Equation 1 vanishes, resulting in $Z(\omega) = Z'(\omega) = R_s$, which is an intercept on the $Z'(\omega)$ axis on the high-frequency side ($\phi = 0$ or $Z''(\omega) = 0$). For $\omega \rightarrow 0$, Equation 1 becomes $Z(\omega) = R_s + R_{et}$, which is an intercept on the $Z'(\omega)$ axis on the low-frequency side. At the frequency where a maximum $Z''(\omega)$ is observed, the straightforward relationship $R_{et} \cdot C_{dl} = 1/\omega_{max}$, which is the time constant of the electrochemical reaction, can be obtained and this indicates how fast the reaction takes place. If $R_{et} \cdot C_{dl}$ is known, C_{dl} can be obtained because R_{et} is already known from the low-frequency intercept on the $Z'(\omega)$ axis. The Nyquist plot gives all the necessary

information about the electrode-electrolyte interface and the reaction (Park and Yoo, 2003).

Similar information can be obtained by examining the Bode diagram (Figure 11) using Equation 1. In this case the logs of amplitudes (absolutes) of the impedance or phase angles are plotted against the log of frequency. Log R_s and log ($R_s + R_{et}$) are obtained straightforwardly from the log $Z(\omega)$ versus log ω plot at high and low frequencies (Figure 11(a)) from the same argument as the Nyquist plot. In the intermediate frequency region, an almost straight line with a slope of ~ -1.0 can be seen. The equation for this line is obtained by ignoring the frequency-independent terms, R_s and 1 in the denominator, of Equation 1 to yield

$$Z(\omega) = R_s + \frac{R_{et}}{1 + j\omega R_{et} C_{dl}} \qquad (2)$$

Taking the logarithm on both sides of the resulting equation yield $\log Z(\omega) = -\log \omega - \log C_{dl}$, which says that $\log|Z(\omega)|$ versus $\log \omega$ would have a slope of -1, and C_{dl} can be obtained from the intercept of this line with the $Z(\omega)$ axis when $-\log \omega = 0$ at $\omega = 1$. Thus, the Bode plot provides the same information as the Nyquist plot. The ϕ (phase angle) versus log ω plot (Figure 11(b)) shows that the impedance responses are resistive primary at high and low frequencies as indicated by practically no phase shifts, whereas at intermediate frequencies, they are mostly capacitive as their phase shifts get closer to 90°(Berney *et al.*, 2000; Park and Yoo, 2003; Wu *et al.*, 2005). Since affinity binding can be detected from the capacitance change at the surface (Wu *et al.*, 2005), the optimum frequency for monitoring the binding reaction based on capacitance change should be the one where the system exhibits near ideal capacitor behavior. This frequency can be obtained from Figure 11(b), i.e. the frequency that provides the phase angle closest to 90°.

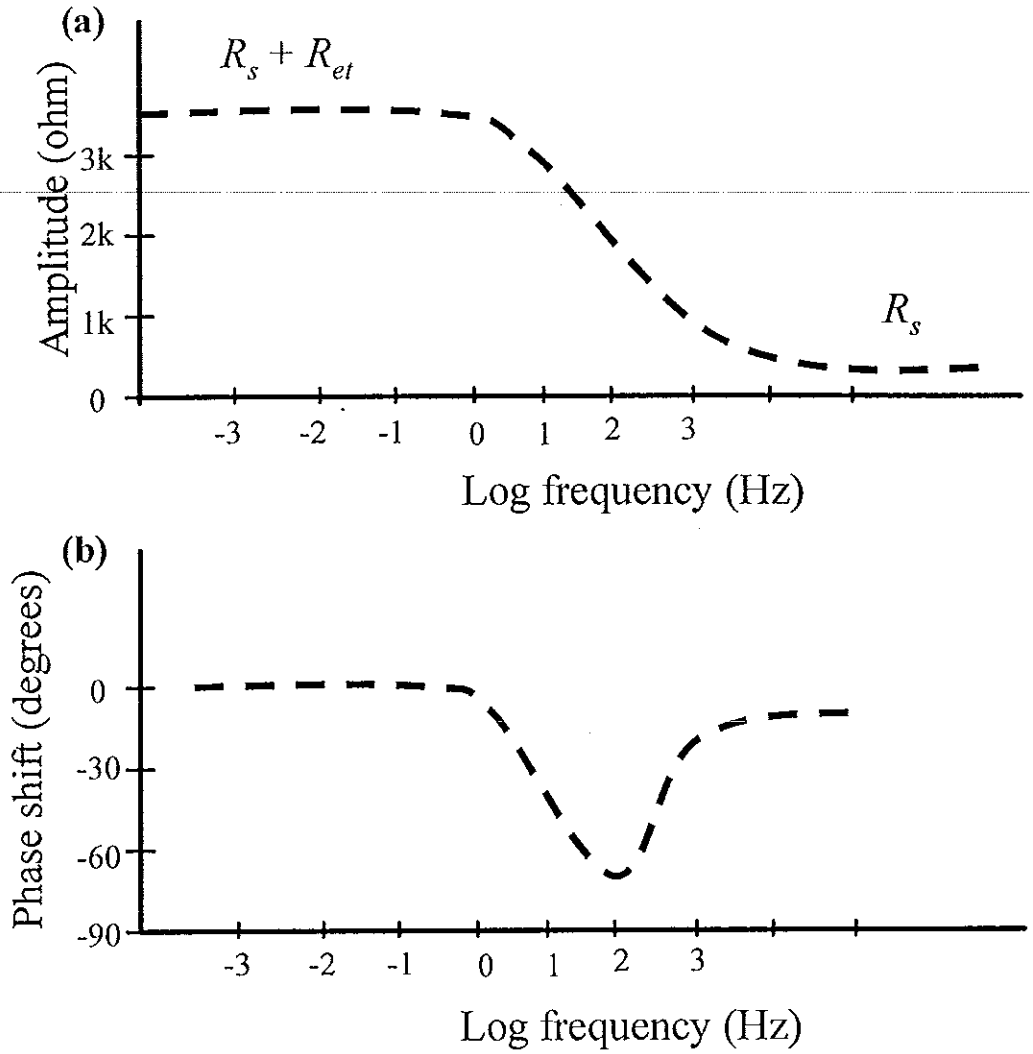


Figure 11 Bode plots, log of amplitudes (absolutes) of the impedance (a) or phase angles are plotted against the log of frequency (b).

Impedance measurement is an effective technique to probe the interfacial properties of antigen-antibody reaction at modified electrode surface, particularly label-free immunosensor (Guan *et al.*, 2004; Wang *et al.*, 2004). In impedimetric immunosensor, antibody was immobilized onto the electrode surface. The change in the electrochemical characteristics took place during the binding of specific antigen. The charge-transfer resistance (R_{et}) calculated from the semicircle in the Nyquist plot increased due to the formation of the stable antigen-antibody complex (Ouerghi *et al.*, 2002; Wang *et al.*, 2004; Hleli *et al.*, 2006).

$$\Delta R_{et} = R_{Ab-Ag} - R_{Ab} \qquad (3)$$

Where $R_{Ab\text{-}Ag}$ is the value of electron-transfer resistance after antigen binding to antibody, R_{Ab} is the value of electron-transfer resistance of the immobilized antibody. However, the data of this imunosensor is difficult to intercept and it is not suitable for real-time measurements (Berggren and Johansson, 1997; Bordi *et al.*, 2002; Guiducci *et al.*, 2004) since the Nyquist plot is done after the signals are collected from the scan frequency range.

To detect the change in real time, impedance due to the capacitance can be monitor. The capacitance changes can be derived from the imaginary part, Z", of the complex impedance spectra. Therefore, the optimal frequency to detect capacitance changes, observed upon formation of the bioaffinity complexes, could be found as described earlier (Bode plot Figure 11(b), frequency at phase angle closest to 90°).

Quantitative analysis can then be measured by monitoring the change of the impedance at a single frequency with time (Dijksma *et al.*, 2001; Bart *et al.*, 2005). Using this principle anti-interferon-γ has been immobilized on electrode surface and when the anti-interferon-γ bound to interferon-γ the impedance Z' and Z" increased with time. The change in impedance can then be obtained from equation 4 (Figure 12(a)) and equation 5 (Figure 12(b)).

$$\Delta Z' = Z'_{Ab-Ag} - Z'_{Ab} \qquad (4)$$

$$\Delta Z'' = Z''_{Ab-Ag} - Z''_{Ab} \qquad (5)$$

Where $Z'_{Ab\text{-}Ag}$ is the value of real part of antigen binding to antibody, Z'_{Ab} is the value of real part of the immobilized antibody, $Z''_{Ab\text{-}Ag}$ is the value of imaginary part of antigen binding to antibody, Z''_{Ab} is the value of imaginary part of the immobilized antibody.

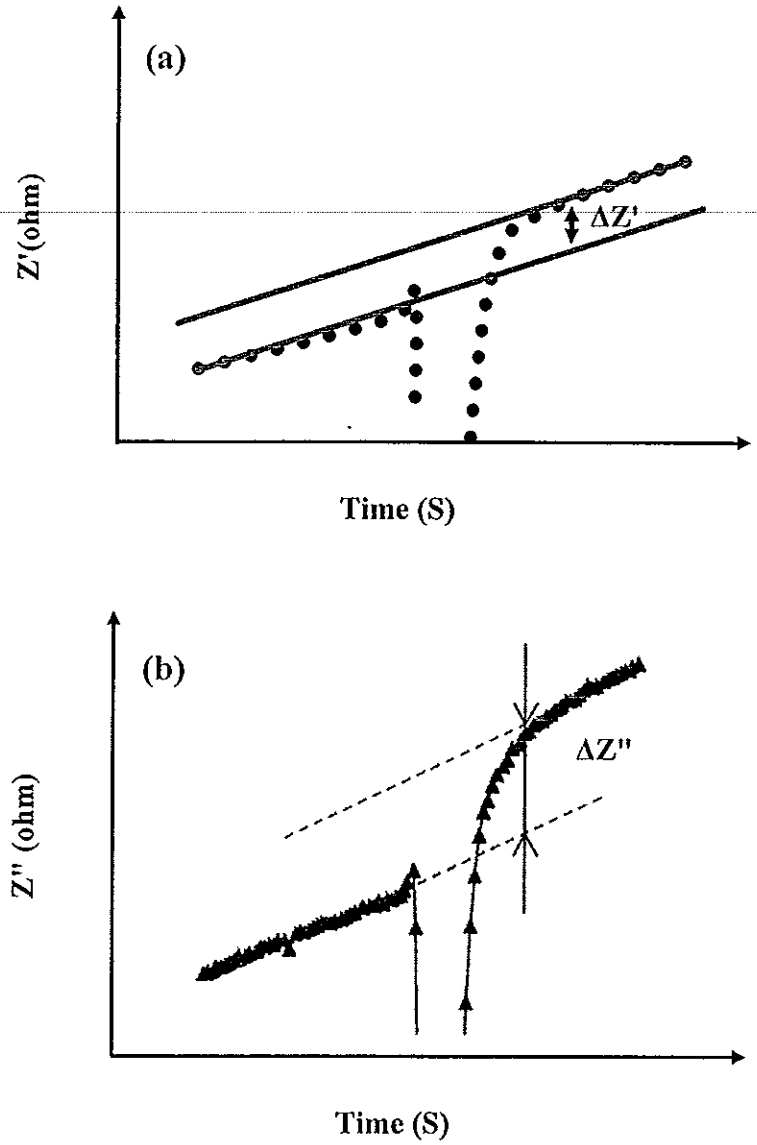


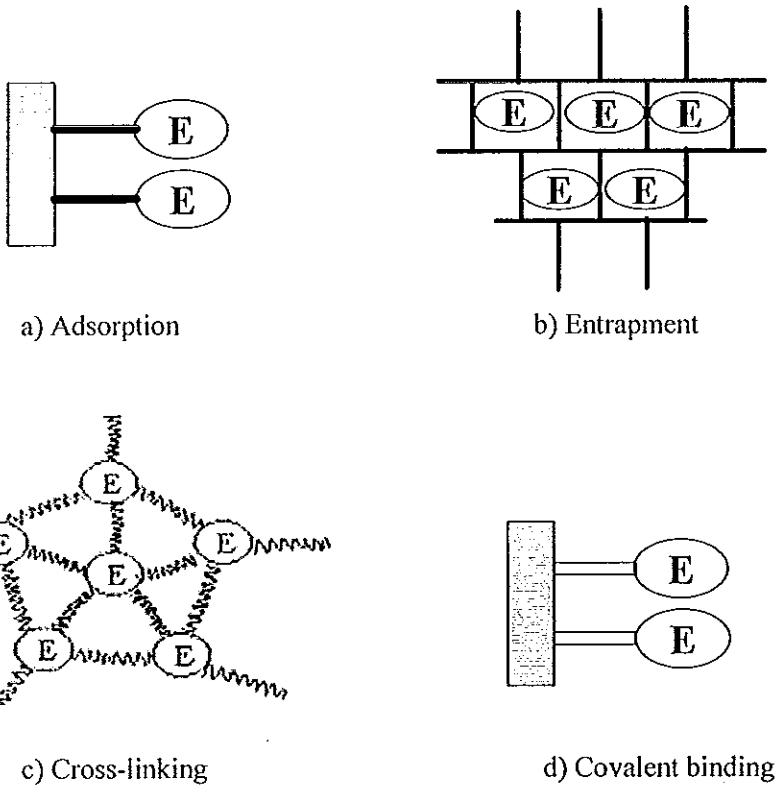
Figure 12 Monitoring of impedance change when antigen-antibody binding, (a) the real part (Z') and (b) the imaginary part (Z'') (modified from Bart *et al.*, 2005).

From the results the increase of Z' did not correlate well with the analyte concentration (Dijksma *et al.*, 2001). Therefore, Z'' was used for quantitative analysis of interferon-γ, where the limits of detection were 10^{-18} M (Dijksma *et al.*, 2001; Bart *et al.*, 2005).

1.4.4 Immobilization

One key factor in biosensor construction is the development of immobilization technologies for stabilizing biomolecules and tethering them to surfaces. The usual aim is to produce a thin film of immobilized biologically active material on or near the transducer surface which responds only to the presence of one or a group of materials or substances requiring detection (Collings and Caruso, 1997).

Various methods are available for immobilization of biomolecules, but not always appropriate for manufacture of biosensors. The most commonly used biomaterial immobilization techniques for designing and development of specific sensors are physical adsorption, entrapment, inter molecular cross-linking and covalent binding as shown in Figure 13 (Sharma *et al.*, 2003).



a) Adsorption b) Entrapment

c) Cross-linking d) Covalent binding

Figure 13 Immobilization methods of biomolecules (modified from Sharma *et al.*, 2003).

For impedimetric detection, a special immobilization technique is required. Since this technique relies of the detection of interfacial changes originating from biorecognition events at electrode surface (Katz and Willner, 2003), therefore, the electrode surface has to be electrically insulated. Thiol based self-assembled monolayer (SAM) is one possible method that can fulfill this requirement (Wrobel, 2001) and it has been used to immobilize antibodies in several reports (Rickert *et al.*, 1996; Dijksma *et al.*, 2001; Limbut *et al.*, 2006). The strong affinity of thiols towards gold surface produces a fast initial adsorption followed by a slower process of reorganization of the S/SH molecules on the gold surface (Yang *et al.*, 1996). This strong affinity and self-assembled facilitates the preparation of structured oriented layers containing functional groups well ordered at the monolayer-air (or liquid) interface (Bain and Whitesides, 1988). SAM is an excellent immobilization technique for protein, it shields protein from direct contact with solid surface, thus reduces the risk of the sensing element denaturation (Wadu-Mesthrige *et al.*, 2000). Therefore, SAM is used for immobilization technique in this work.

1.5 Objective

To determine penicillin G in milk by impedimetric immunosensor.

1.6 Benefits

It is expected that the developed impedimetric biosensor technique which is label-free, reusable, sensitive and requires relatively short analysis time will be used as an alternative approach for determining antibiotic in food.

1.7 Outline of the research

1. Immobilize antibody via self-assembled monolayer.

2. Optimize the operating conditions of a flow injection impedimetric immunosensor for penicillin G, such as flow rate, type of buffer, *etc.* The performance criteria are considered by balancing between impedance changes ($\Delta Z''$) and analysis time

3. Test the performance of impedimetric immunosensor system under the optimum conditions, such as linear range, LOD and specificity.

4. Test the impedimetric immunosensor system under the optimum condition by determining penicillin G in milk samples.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

- Anti-penicillin G (IgG1, k; USBiological, USA)
- Penicillin G, sodium salt ($C_{16}H_{17}N_2O_4NaS$; USBiological, USA)
- Gold electrode (mean diameter 3.0 mm, 99.99% purity)
- Hydrogenperoxide (H_2O_2; Merck, Germany)
- Sulfuric acid (H_2SO_4; AR Grade: LAB-SCAN, Thailand)
- Thioctic acid ($C_8H_{14}O_2S_2$; Sigma, USA)
- *N*–(3-Dimethylaminopropyl)-*N*-ethyl carbodiimide hydrochloride (EDC; $C_8H_{17}N_3HCl$; Sigma, USA)
- *N*-hydroxysuccinimide 97 % (NHS; $C_4H_5NO_3$; Sigma, USA)
- Sodium azide (NaN_3; AR Grade: Merck, Germany.)
- 1-dodecanethiol 98+% ($CH_3(CH_2)_{11}SH$; ALDRICH, USA)
- Potassium dihydrogenphosphate dihydrate ($KH_2PO_4 \cdot 2H_2O$; AR Grade: Merck, Germany.)
- Dipotassium hydrogenphosphate dihydrate ($K_2HPO_4 \cdot 2H_2O$; AR Grade: Merck, Germany.)
- Glycine (H_2NCH_2COOH; AR Grade: Merck, Germany.)
- Sodium hydroxide (NaOH; AR Grade: Merck, Germany.)
- Sodium chloride (NaCl; AR Grade: BDH, England.)
- Potassium chloride (KCl; UNINAR, Australia.)
- Hydrochloric acid 36.5-38 % (HCl; AR Grade: BDH, England.)
- Ampicillin trihydrate ($C_{16}H_{19}N_3O_4S \cdot 3H_2O$; Sigma, USA)
- Amoxacillin trihydrate (Bureau of Drug and Narcotics, Department of Medical Sciences, Ministry of Public Health, Thailand)
- Cloxacillin (Bureau of Drug and Narcotics, Department of Medical Sciences, Ministry of Public Health, Thailand)
- Ciprofloxacin HCl (Bureau of Drug and Narcotics, Department of Medical Sciences, Ministry of Public Health, Thailand)

- Ofloxacin (Bureau of Drug and Narcotics, Department of Medical Sciences, Ministry of Public Health, Thailand)
- Alumina polishing powder diameter 5.0, 1.0 and 0.3 μm (Metkon, Turkey)
- Ethanol (C_2H_5OH; Merck, Germany)
- Methanol (CH_3OH; Merck, Germany)
- Acetone (CH_3COCH_3; AR Grade, BDH, England)
- Potassium ferricyanide ($K_3Fe(CN)_6$; Sigma, USA)

2.2 Apparatus

- Peristaltic pump (Minipulse3, Gilson, France.)
- Autolab PGSTAT30 electrochemical impedance analyzer and potentiostat/galvanostat (Eco Chemmie, Netherlands)
- Sample injector (VIGO 6 port W valves , Valco, USA.)
- Syringe (NIPRO disposable syringe, Thailand.)
- Computer(LASER, Thailand)
- Polishing machine (Gripo® 2V, Metkon Instruments Ltd., Turkey)
- Vacuum (EDWARDS, England)
- Alber® nylon membrane filter (Albet, Spain) pore size 0.20 μm
- Shaker (Model SK-101; HL, Thailand)
- Microliter pipette 100 μl, 1,000 μl and 5,000 μl (Eppendorf, Geramany)
- Microtubes1.5 ml and 2.0 ml (Axygen, USA)
- Microcentrifuge (Spectrafuge 16 M, Labnet, USA)

2.3 Impedimetric biosensor system

Monoclonal anti-Penicillin G was used as the biological recognition element specific to penicillin G and was immobilized on gold electrode surface via a self-assembled thioctic acid monolayer. When penicillin G bound to the anti-Penicillin G immobilized on the gold working electrode the impedance increased and the response was detected as the change of impedance.

A flow injection impedimetric biosensor system is shown in Figure 14. It consists of

a) A peristaltic pump, where a 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 and an electrochemical detection system, Autolab PGSTAT30 electrochemical impedance analyzer and potentiostat/galvanostat (Eco Chemmie, Netherlands) connected to a computer. Eco Chemie software with Frequency Response Analyzer (FRA 4.9.005) was used to monitor impedance change of interfacial properties of the electrode. The reaction flow cell (Figure 15) consisted of a modified gold working electrode, a laboratory built Ag/AgCl reference electrode and a stainless steel tube, used as an auxiliary electrode and outlet.

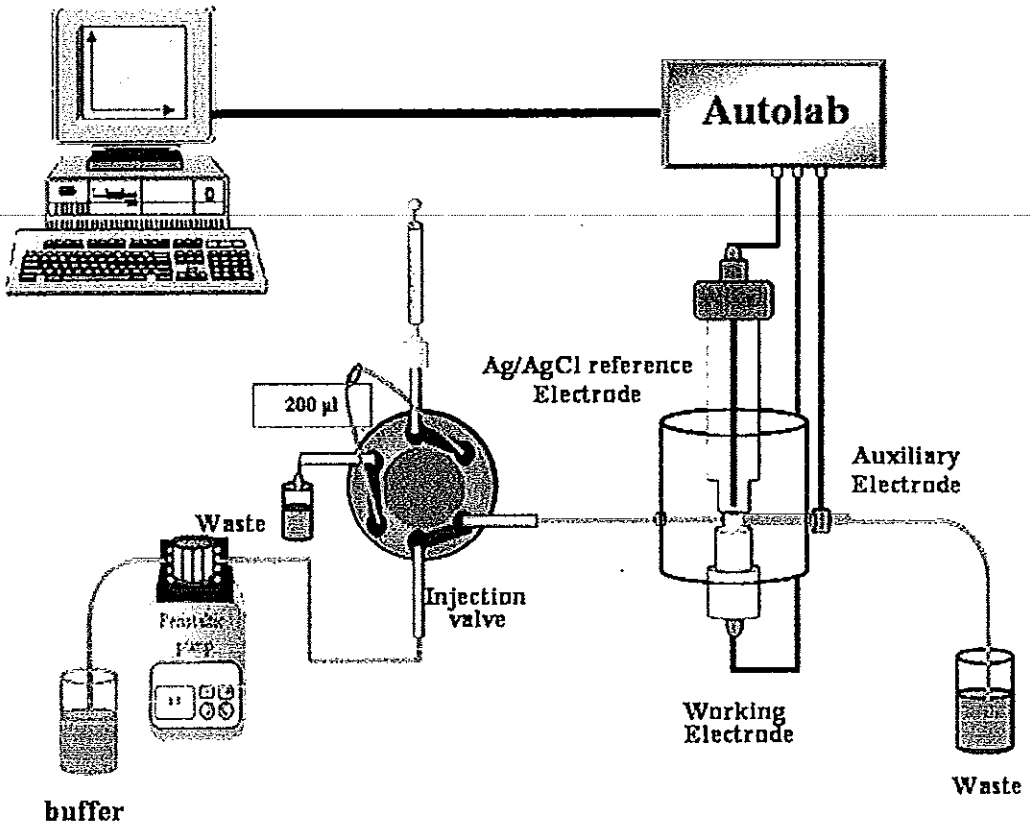


Figure 14 Schematic diagram showing the impedimetric biosensor system (Modified from Limbut, 2006).

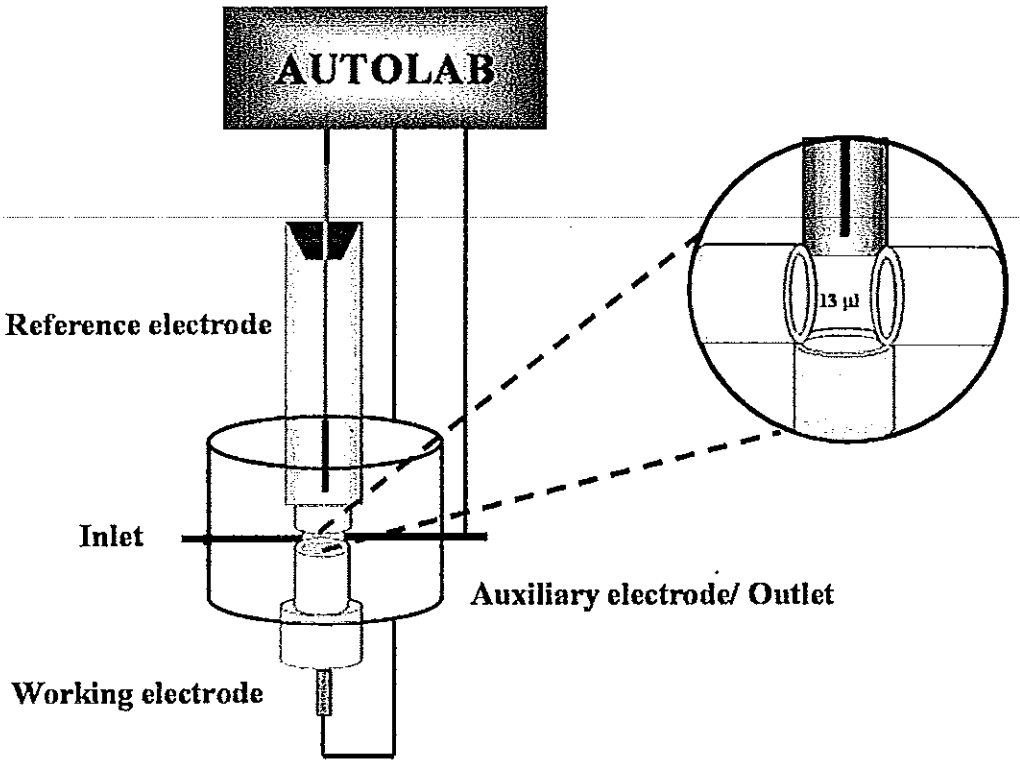


Figure 15 Schematic diagram showing the reaction flow cell (Modified from Limbut, 2006).

2.4 Modified gold electrode

2.4.1 Preparation of gold surface

A gold electrode (Ø 3.0 mm, 99.99% purity) was used as the working electrode in the three electrodes electrochemical detection system. Gold surface was polished by a polishing machine (Gripo® 2V, Metkon Instruments Ltd., Turkey) using alumina slurries with particle diameters 5.0, 1.0 and 0.3 µm (Metkon, Turkey) respectively, and subsequently rinsed with DI water and absolute ethanol. It was cleaned through sonication in DI water for 15 min, followed by electrochemical etching in 0.5 M H_2SO_4 using cycling electrode potential from 0 to 1.5 V versus Ag/AgCl reference electrode with a scan rate of 0.1 Vs^{-1} for 25 scans (Autolab PGSTAT30). The electrode was then dried with pure nitrogen gas.

2.4.2 Immobilization of anti-Penicillin G

The cleaned gold electrodes were immediately put in thioctic acid solution (250 mM in absolute ethanol). After 12 h the electrodes were thoroughly rinsed with absolute ethanol and DI water then dried with pure nitrogen gas. In this step self-assembled thioctic acid monolayer was formed on gold surface (Figure 16 (1)). Thereafter, the gold electrodes were put in EDC: NHS solution (EDC 1% (v/v), NHS 2.5%(v/v) in 0.05 M phosphate buffer pH 5.00 with 0.05 M KCl) for 5 h where the terminal OH group of the thiol was activated. *N*-substituted carbodiimides can react with a carboxylic group to form a highly reactive *O*-acylisourea which then reacts with primary amine. (Hermanson *et al.,* 1992; Vaughan *et al.,* 1999) (Figure 16 (2)). The electrodes were rinsed with DI water and dried with pure nitrogen gas. Then 20 µl of 0.25 mg ml^{-1} anti-Penicillin G in phosphate buffer, pH 7.40 was placed on the modified surface (Figure 16 (3)) and left for the reaction to take place overnight at 4°C. The terminal amine group of antibody displaces the NHS group and thus binds covalently to the monolayer (Vaughan *et al.,* 1999). Finally, the electrodes were reacted in a 10 mM 1-dodecanethiol ethanolic solution for 30 min to block any pinholes or bare spots on the electrode surface (Figure 16 (4)).

During the immobilization steps, the electrochemical characteristics of modified electrodes were studied using cyclic voltammetry. These measurements were performed in an unstirred solution of 0.05 M $K_3[Fe(CN)_6]$ with 0.1 M KCl using an electrochemical cell equipped with three electrodes system consisted of immobilized anti-Penicillin G gold electrode as working electrode, Ag/AgCl reference electrode and a platinum wire as auxiliary electrode (Figure 17). These scans were performed from -0.3 to 0.7 V versus Ag/AgCl reference electrode with a scan rate of 0.1 Vs^{-1}.

After prolong use the activity of anti-Penicillin G would decline. The immobilized layer on the surface can then be removed by sonication in oxidizing agent (H_2O_2 : H_2SO_4, 1 : 3) for 15 min. The gold electrode can then be reused.

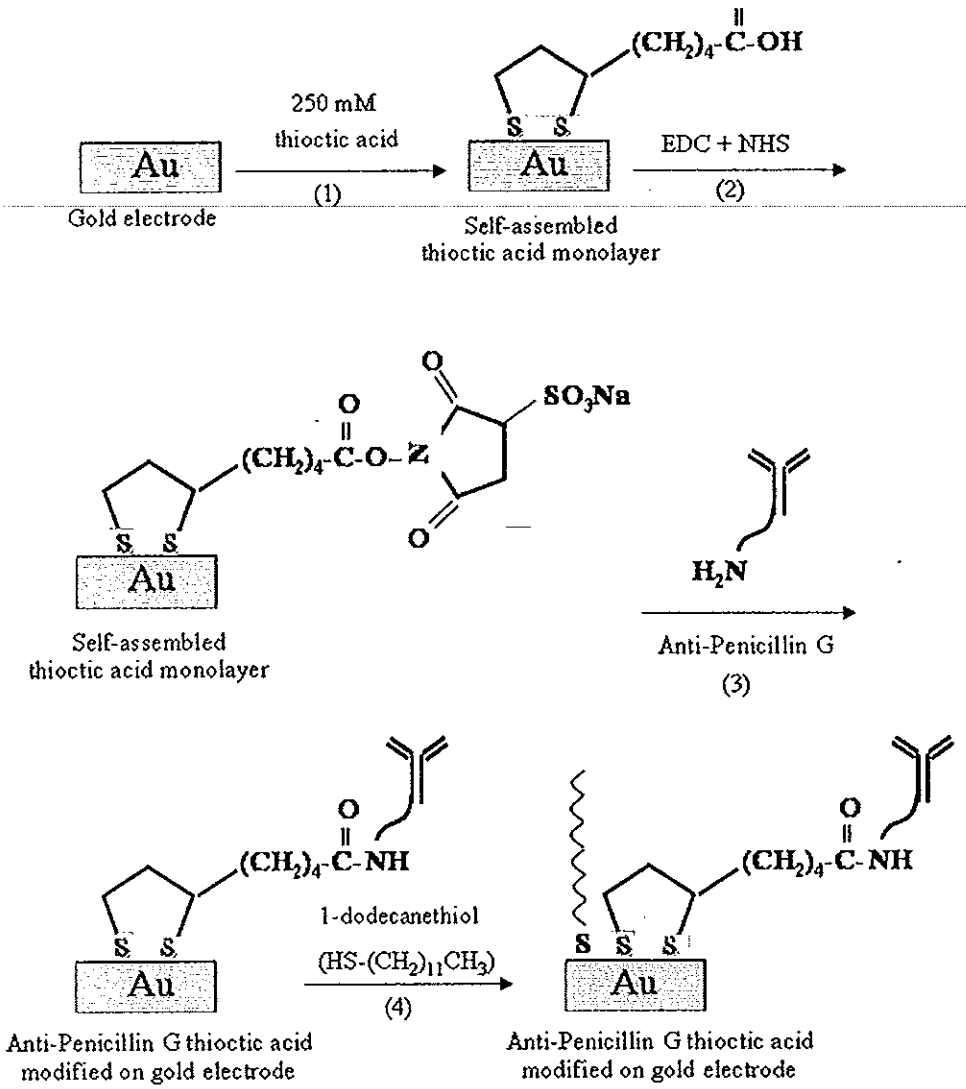


Figure 16 Reaction mechanisms during the immobilization steps of the anti-Penicillin G immobilized on a self-assemble thioctic acid monolayer (Modified from Limbut, 2006).

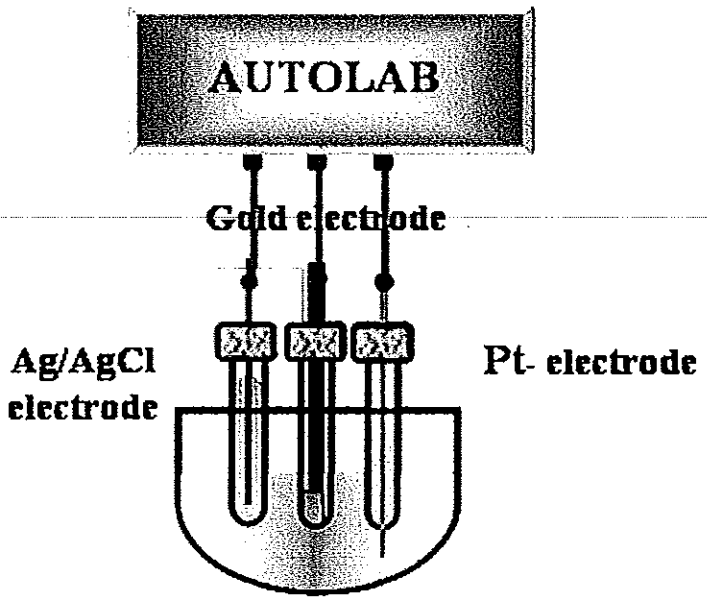


Figure 17 Schematic diagram showing the cyclic voltammetry system (Modified from Limbut, 2006).

2.5 Impedimetric measurement

A flow-injection (FI) technique was applied to determine penicillin G in an impedimetric biosensor system (alternative current voltage at 0.01 V amplitude). The electrochemical measurements were performed by using Autolab PGSTAT30 electrochemical impedance analyzer and potentiostat/ galvanostat (Eco Chemmie, Netherlands) connected to a computer. Eco Chemie software, Frequency Response Analyzer (FRA 4.9.005), was used to monitor the impedance.

To investigate the change of impedance we chose to monitor the imaginary part of the impedance (Z") with time since the change of Z" provides better correlation to analyte concentration than the real part of impedance (Z') (Bart *et al.*, 2005). Since the impedance depends on the frequency (Bart *et al.*, 2005) it is necessary to find the frequency that will provide the best response. The optimum frequency for monitoring the immunoreactions was determined by considering the effect of frequency on impedance and phase angle. Phase angle and logarithm of impedance magnitude (the absolute impedance) were plotted *vs.* the logarithm of

frequency (Figure 11, 1.4.3). 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° as shown in Figure 18. At this frequency the system exhibits near ideal capacitor behavior (the imaginary part of impedance, Z″) (Berney *et al.*, 2000: Wu *et al.*, 2005).

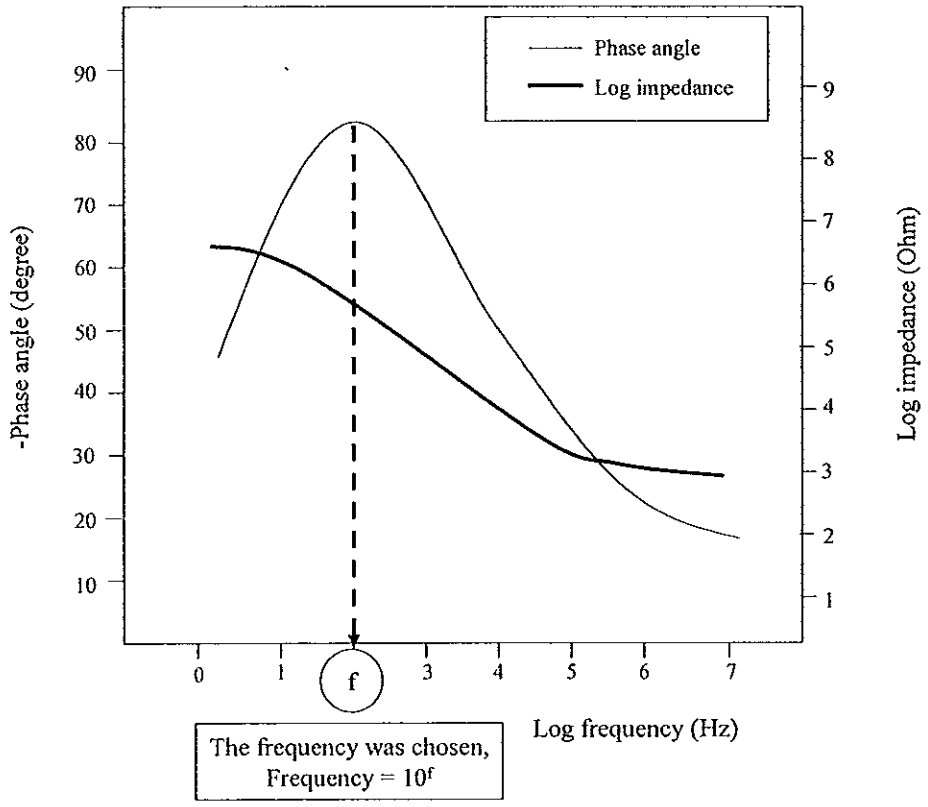


Figure 18 Schematic diagram showing the Bode plot for determination of frequency (Modified from Wu *et al.*, 2005).

The optimum frequency was used to monitor the impedance change with time (Dijksma *et al.*, 2001; Bart *et al.*, 2005). When the analyte (antigen) bind to the immobilized antibody on the working electrode the impedance increases and the impedance change (ΔZ″) can be determined as shown in Figure 19. The interaction

between analyte and antibody on electrode surface are non-covalent and the analyte can be dissociated from the antibody by using regeneration solution.

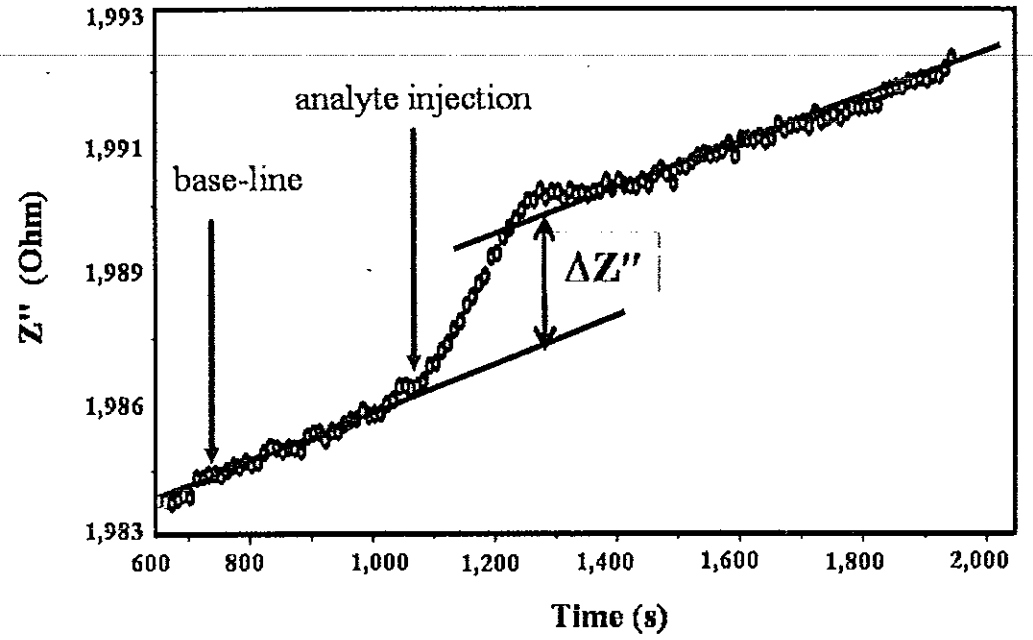


Figure 19 Diagram showing the change in impedance (ΔZ″) as a function of time caused by binding between antigen and antibody.

2.6 Optimization of operating conditions

Operating conditions of impedimetric biosensor in a flow injection analysis system were optimized. These included flow rate, type and pH of buffer solution, sample volume, type, pH, and concentration of regeneration solution. The optimum of each parameter was considered by balancing between impedance change (ΔZ″) and analysis time. Optimizations were carried out by varying one parameter at a time while the others were kept constant. In these studies, 1.0×10^{-11} M standard penicillin G in 10 mM phosphate buffer pH 7.40, was injected. Three replications were performed for each tested value. The starting operating conditions of impedimetric biosensor system were shown in Table 2. When the optimum value of one parameter was obtained it was used for the optimization of the next parameter following the sequence in this section.

Table 2 Starting operating conditions.

Condition	Value
1. Flow rate (μl min^{-1})	200
2. Type of buffer solution	10 mM phosphate buffer pH 7.40
3. pH of buffer solution	10 mM phosphate buffer pH 7.40
4. Sample volume (μl)	200
5. Type of regeneration solution	Low pH (acid): HCl
6. pH of regeneration solution	HCl pH 2.60

2.6.1 Flow rate

In a flow system the flow rate of the buffer passing through the flow cell is the main factor affecting the yield of interaction between penicillin G and immobilized anti-Penicillin G on the gold electrode surface. So optimization of flow rate is necessary. The effect of the flow rate was studied at 50, 100, 150, 175, 200, 250 and 300 μl min^{-1}.

2.6.2 Type of buffer solution

Buffers that have been used in impedimetric biosensor systems were tested, i.e., 10 mM phosphate buffer solution (PBS) pH 7.4 + 0.1 M KCl + 2.5 mM $Fe(CN)_6$ (Tang *et al.*, 2004), 10 mM phosphate buffer saline pH 7.4 (Maupas *et al.*, 1997) and tris-buffered (Wang *et al.*, 2005; Wang *et al.*, 2006). Preliminary test using 10 mM phosphate buffer solution (PBS) pH 7.4 + 0.1 M KCl + 2.5 mM $Fe(CN)_6$ gave unstable signal since $Fe(CN)_6$ was found deposited at the reference electrode surface. Therefore, the effect of type of buffer solution was only compared between phosphate buffer saline (PBS) and tris-HCl.

2.6.3 pH of buffer solution

The influence of pH during binding reaction was studied using 10 mM phosphate buffer pH 6.80, 7.00, 7.20, 7.40 and 7.60 (optimum type of buffer solution in 2.6.2).

2.6.4 Sample volume

One way of improving the response of the system is by increasing the analyte sample volume. However, in affinity biosensor system, antigen-antibody binding also depends on the amount of antibody immobilized on electrode surface. So, too much analyte for the same amount of antibody can not increase the response. Therefore, a suitable sample volume should be found. The tested volumes were 50, 100, 150, 175, 200, 250 and 300 µl. The flow rate of the buffer was 200 µl min^{-1} (optimum found in 2.6.1).

2.6.5 Type of regeneration solution

To ensure reproducible measurements, a complete regeneration of the surface between sample injections must be obtained without affecting the characteristics of ligand. Ideally the regeneration solution should remove all compounds that bind non-covalently to surface and the binding capacity of the surface should not be affected by the regeneration and the baseline should remain at constant level. The most commonly used regeneration solutions are agents with high ionic strength, low or high pH (Andersson *et al.*, 1999).

The effect of regeneration solution was studied using solutions with

(i) high ionic strength; 1.0 M NaCl, 1.0 M KCl, 1.0 M $MgCl_2$,

(ii) low pH; 0.1 M glycine-HCl pH 2.50 in 0.4 M NaCl, 0.1 M glycine-HCl pH 2.50, HCl pH 2.5 and

(iii) high pH; 0.005 M NaOH and 0.05 M NaOH.

2.6.6 pH of regeneration solution

The pH of the best regeneration solution found in 2.6.5, glycine-HCl, was studied at 2.00, 2.10, 2.20, 2.30, 2.40, 2.50, 2.60, 2.70, 2.80, 2.90 and 3.00.

2.6.7 Concentration of regeneration solution

The concentration of regeneration solution is important for the stability of electrodes and this was studied at 10, 25, 40, 50, 75 and 100 mM glycine-HCl

buffer pH 2.50 (optimum found 2.6.5 and 2.6.6). The tested values for the optimization of operating parameters are summarized in Table 3.

Table 3 Tested values of the operating conditions.

Condition	Tested value
1. Flow rate (μl min^{-1})	50, 100, 150, 175, 200, 250 and 300
2. Type of buffer solution	10 mM phosphate buffer pH 7.40 and 10 mM Tris-HCl buffer pH 7.40
3. pH of buffer solution	10 mM phosphate buffer pH 6.80, 7.00, 7.20, 7.40 and 7.60
4. Sample volume (μl)	50, 100, 150, 175, 200, 250 and 300
5. Type of regeneration solution	High ionic strength - 1.0 M NaCl, - 1.0 M KCl and - 1.0 M $MgCl_2$ Low pH (acid) - 0.1 M glycine-HCl pH 2.50 in 0.4 M NaCl - 0.1M glycine-HCl pH 2.50 and - HCl pH 2.50 High pH (base) - 0.005 M NaOH and - 0.05 M NaOH
6. pH of regeneration solution	100 mM glycine-HCl pH 2.00, 2.10, 2.20, 2.30, 2.40, 2.50, 2.60, 2.70,2.80, 2.90 and 3.00
7. Concentration of regeneration solution	10, 25, 40, 50, 75 and 100 mM glycine-HCl pH 2.50

2.7 Reproducibility

In this work, 50 mM glycine-HCl buffer solution, pH 2.50 was chosen to break the binding between Penicillin G and anti-Penicillin G. Penicillin G was detected by regenerating the electrode 15 times per day for about 3-4 days. The reproducibility of anti-Penicillin G modified electrodes were evaluated by monitoring the impedance change ($\Delta Z''$) at the same concentration of standard penicillin G (1.0×10^{-11} M) at a flow rate of 200 µl min^{-1} phosphate buffer solution, pH 7.40 and a sample volume of 200 µl.

2.8 Selectivity

The effect of substances that might interfere with the response of the Penicillin G in the impedimetric biosensor system was studies. Two groups of antibiotic, having similar physical and chemical characteristics to penicillin G, i.e., the penicillin group such as ampicillin, amoxacillin trihydrate and cloxacillin, and the beta-lactam group such as ciprofloxacin HCl and ofloxacin were used to test the selectivity of the impedimetric biosensor system.

2.9 Linear dynamic range, sensitivity and detection limit

After penicillin G was injected into the impedimetric biosensor system it binds to anti-Penicillin G immobilized on the surface of gold electrode. A calibration curve is plotted between the response change ($\Delta Z''$) and the logarithm of analyte concentration (Bart *et al.,* 2005). The linear range is the interval between the upper and lower levels of the analyte concentration that have been demonstrated to be determined with linearity. Sensitivity is determined within the linear concentration range of the immunosensor calibration curve. The sensitivity is the slope of the calibration curve, i.e., response change/analyte concentration ($\Delta R/\Delta C$) or response change/logarithm of analyte concentration ($\Delta R/$ (log C)) (Thévenot *et al.*, 1999; 2001) (Figure 20).

The limit of detection (LOD) is defined as the lowest concentration of analyte in a sample that can be detected, though not necessarily quantitated (Swartz and Krull, 1997). There are several methods to evaluate LOD (Long and Winefordner,

1982; Miller and Miller, 1993; Taverniers *et al.*, 2004). Because of the characteristic of the responses, the LOD of the work presented in this thesis follows the IUPAC Recommendation 1994 (Buck and Lindner, 1994). It is defined as the concentration of the analyte at which the extrapolated linear portion of the calibration curve intercepts the baseline-a horizontal line corresponding to zero change in response over several decades of concentration change (Figure 20).

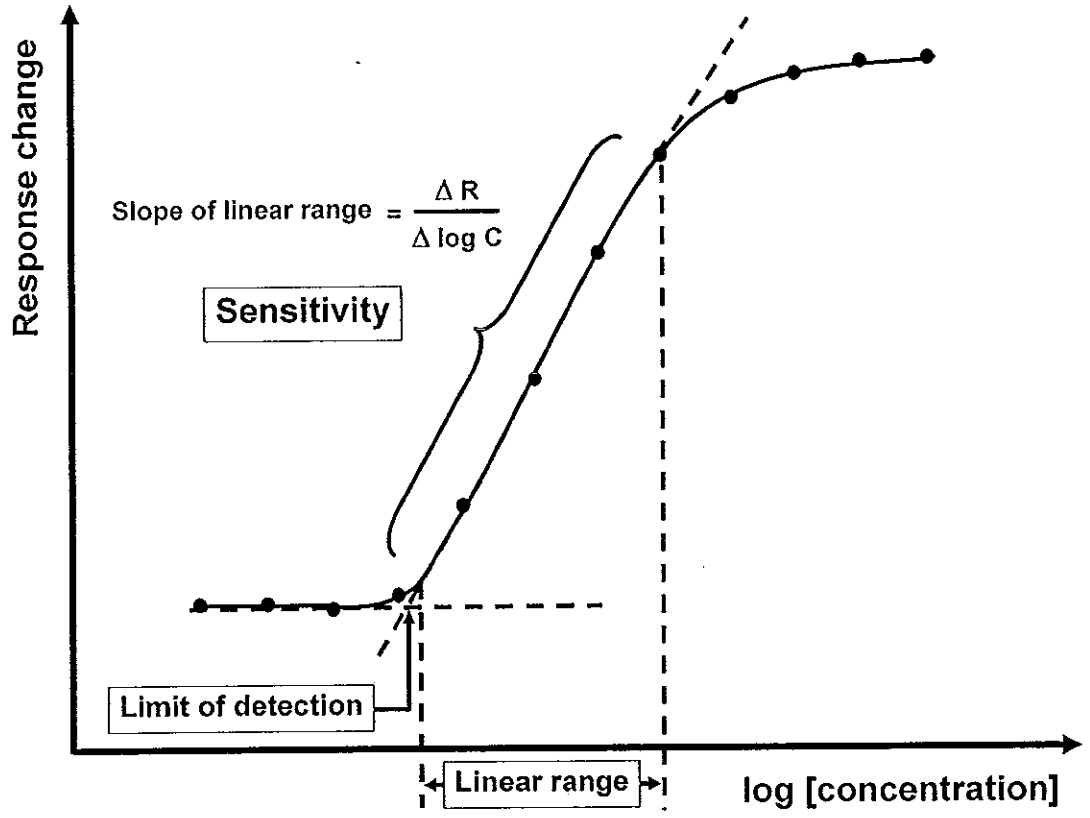


Figure 20 A calibration curve showing relationships for determining linear range, sensitivity and limit of detection (Buck and Lindner, 1994; Eggins, 1996; Swartz and Krull, 1997; Thevenot *et al.*, 1999)

2.10 Determination of Penicillin G in milk samples

To demonstrate the use of the impedimetric biosensor, raw milk samples obtained from Department of Animal Science, Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Thailand and pasteurized milk from the store were tested.

Milk samples were diluted by 10, 20, 40, 100 and 10,000 times using 10 mM phosphate buffer pH 7.40 and used to determine the recovery and matrix effect. The sample solutions were injected into the impedimetric biosensor system. The change in the impedance of each sample was used to calculate penicillin G concentration using calibration curve done prior to the test.

2.10.1 Validation of method

Validating a method is investigating whether the analytical purpose of the method is achieved, which is obtaining analytical result with an acceptable uncertainly level (Taverniers *et al.*, 2004). The objective of the method validation is to demonstrate that the impedimetric biosensor can be used to detect penicillin G in milk samples with acceptable accuracy. Three milk samples were used in the study to validate the method.

2.10.2 Recovery

The recovery of the method was tested at the concentrations close to the maximum residues limit (MRL) of penicillin G, 1.0×10^{-8} M (EU). This was performed by spiking milk samples with standard penicillin G, 1.0×10^{-9}, 5.0×10^{-9}, 1.0×10^{-8}, $5.0 \times 10^{-8}$and 1.0×10^{-7} M. The spiked milk samples were diluted 10,000 times with 10 mM phosphate buffer pH 7.40 and injected into the impedimetric biosensor system. Three replicates were done for each concentration. The results from the spiked milk samples were compared with standard solution prepared at the same concentrations. Percentage recovery (%R) is calculated as follows:

$$\%R = [(CF-CU)/CA] \times 100$$

where CF is the concentration of analyte measured in the spiked sample.

CU is the concentration of analyte measured in the blank.

CA is the concentration of analyte spike in the sample.

(Eurachem, 1998)

2.10.3 Precision

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the percent relative standard deviation for a statically significant number of samples (Swartz *et al.*, 1997). To assess the precision of the method three milk samples and 1.0 x 10^{-11} M standard penicillin G were evaluated. The milk samples and standard penicillin G were prepared with 10 mM phosphate buffer pH 7.40 and injected into the impedimetric biosensor system. Fives replicates were done for each sample and forty-five replicates for the standard penicillin G. The relative standard deviations (RSD) were then calculated for each type of samples by the following equations (Miller and Miller, 2000).

$$\%RSD = \frac{s}{x} \times 100$$

$$s = \sqrt{\frac{\sum\left(x_i - \bar{x}\right)^2}{n-1}}$$

where s is the standard deviation.

n is the total number of measurements.

$\bar{x}$ is the mean of n measurement.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Electrochemical performance of the immobilization process

To confirm that thioctic acid and anti-Penicillin G were successfully assembled on gold working electrode, cyclic voltammetry experiments were performed. The electrochemical characteristics of $K_3[Fe(CN)_6]$ on the modified electrode are shown in Figure 21. When the electrode surface had been modified by thioctic acid and anti-Penicillin G the electron transfer kinetics of $K_3[Fe(CN)_6]$ redox reactions decreased. Figure 21 shows the reduction of redox peaks of $K_3[Fe(CN)_6]$ after each step of the immobilization process. Curve a is the electrochemical response of the redox probe $K_3[Fe(CN)_6]$ on bare gold electrode, curve b is when thioctic acid was self-assembled on gold electrode, curve c is after anti-Penicillin G was immobilized on the gold electrode via thioctic self-assembled and curve d is when pinholes on the electrode surface were blocked by treating with 1-dodecanethiol. The flatness of curve d indicates that the surface is totally insulated, a necessary condition for impedimetric detection.

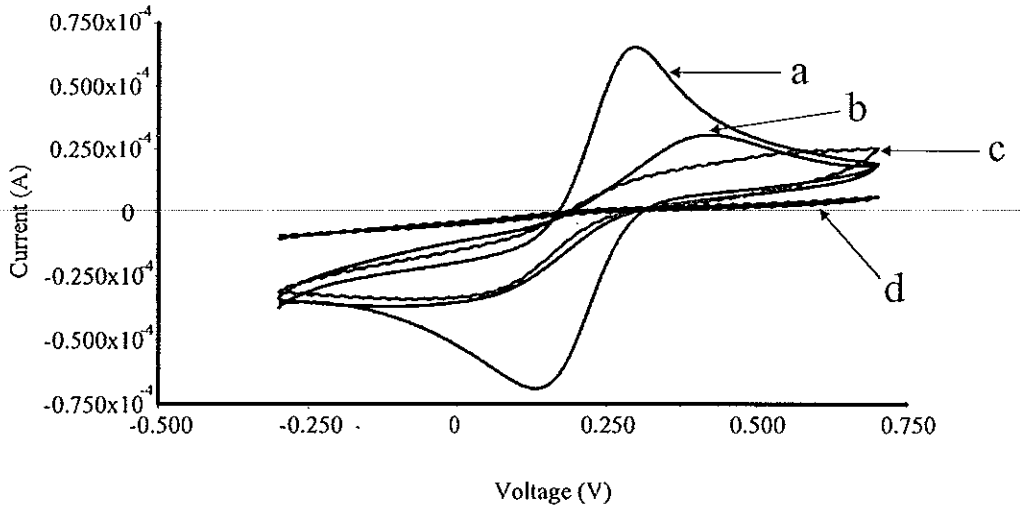


Figure 21 Cyclic voltammograms of gold electrode obtained in 0.05 M $K_3[Fe(CN)_6]$ with 0.1 M KCl, (a) bare gold, (b) thioctic acid covered gold, (c) anti-Penicillin G modified thioctic acid couple gold, and (d) as in (c) but after 1-dodecanethiol treatment.

3.2 Impedimetric measurement

Since impedance depends on frequency, the optimum frequency for monitoring the immunosensors was determined. This was done by applying an alternative current voltage of 0.01 V amplitude at frequency between 0.01 and 10,000 Hz. The effect of frequency can be shown by a Bode plot of phase angle and impedance magnitude on log scale vs. the log of frequency (Figure 22). In the region where the impedance curve is a straight line with slope about -1 and the phase angle as close to -90° as possible the system exhibits near ideal capacitor behavior. The frequency can be found as shown in Figure 22. Several electrodes were tested and the optimum frequency was 160 ± 14 Hz (n = 11), and this mean value was used as the working frequency to monitor the impedance (Z"). This optimum frequency is not the same for different systems. For example in our earlier work (using the same size electrode-3.0 mm diameter) the optimum frequency for anti human serum albumin and albumin interaction was 100 Hz. For a smaller diameter electrode (1.0 mm) Dijksma *et al.* (2001) and Bart *et al.* (2005) used 113 Hz when the gold working electrode was modified with *N*-acetyl-L-cystein and immobilized anti-interferon-γ.

However, with the same size of electrode optimum frequency was at 34 Hz when the gold electrode was modified by polymerization (tyramine) and immobilized anti-HSA (Wu *et al.*, 2005). This is, different optimum frequency may depend on the different modified method, area of the electrode and biological elements.

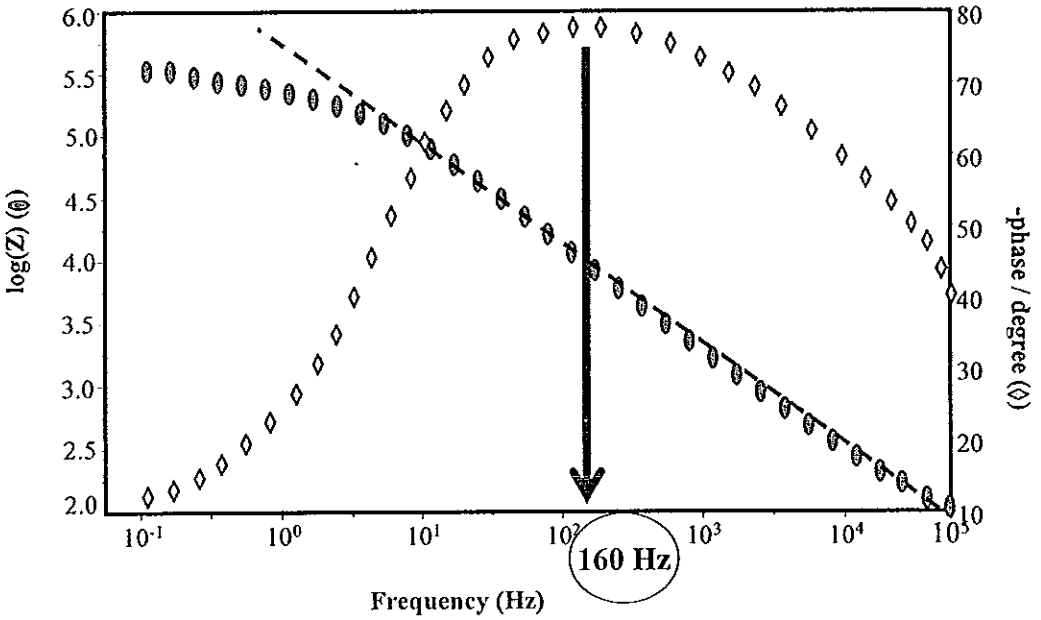


Figure 22 A Bode plot, log of amplitudes (absolutes) of the impedance (0) and phase angles (◊) are plotted against the frequency, to determination of frequency.

Figure 23 shows an example of the response obtained from real time monitoring of Z" at 160 Hz in a flow system. When the buffer solution flowed through the system a baseline was obtained. When the antigen (penicillin G standard solution) was injected it bound to antibody causing the Z" to increase where the change in impedance (ΔZ") could be obtained as shown in Figure 23. Analysis time was the time taken from the point where the response started to increase until it reached a steady value (Figure 23) and this was found to be between 10-12 min depending on the concentration of the analyte. The regeneration solution was then injected to remove the bound antigen from the electrode surface. This was followed by a continuous flow of buffer. When the response reached a baseline the electrode

can be used for the next analysis. The time required to regenerate the electrode surface was 10-12 min.

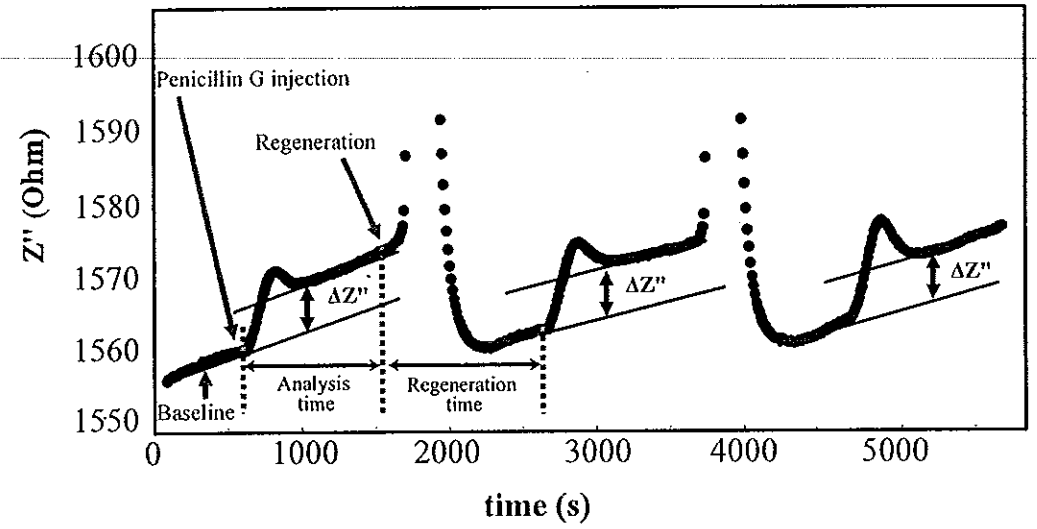


Figure 23 Change of impedance (ΔZ") as a function of time caused by binding of penicillin G and anti-Penicillin G with subsequent regeneration.

3.3 Optimization of operating condition

Primary test for the detection of penicillin G by the immobilized anti-Penicillin G provided the responses as shown in Table 4 and Figure 24. Penicillin G at 1.0×10^{-11}M standard solution, the mid range concentration of the calibration curve, was then applied for the optimization of operating conditions.

Table 4 Responses of a flow injection impedimetric biosensor system for the primary test between penicillin G with anti-Penicillin G binding.

Concentration of penicillin G (M)	ΔZ" (Ohm), mean ± SD*
1.0×10^{-14}	2.8 ± 0.4
1.0×10^{-12}	5.3 ± 0.2
1.0×10^{-10}	8.4 ± 0.2
1.0×10^{-08}	9.8 ± 0.2
1.0×10^{-06}	10.3 ± 0.8
Sensitivity (Ohm/log M) of penicillin G, 1.0×10^{-14}-1.0×10^{-08} M	1.205
r	0.991

* 3 replications

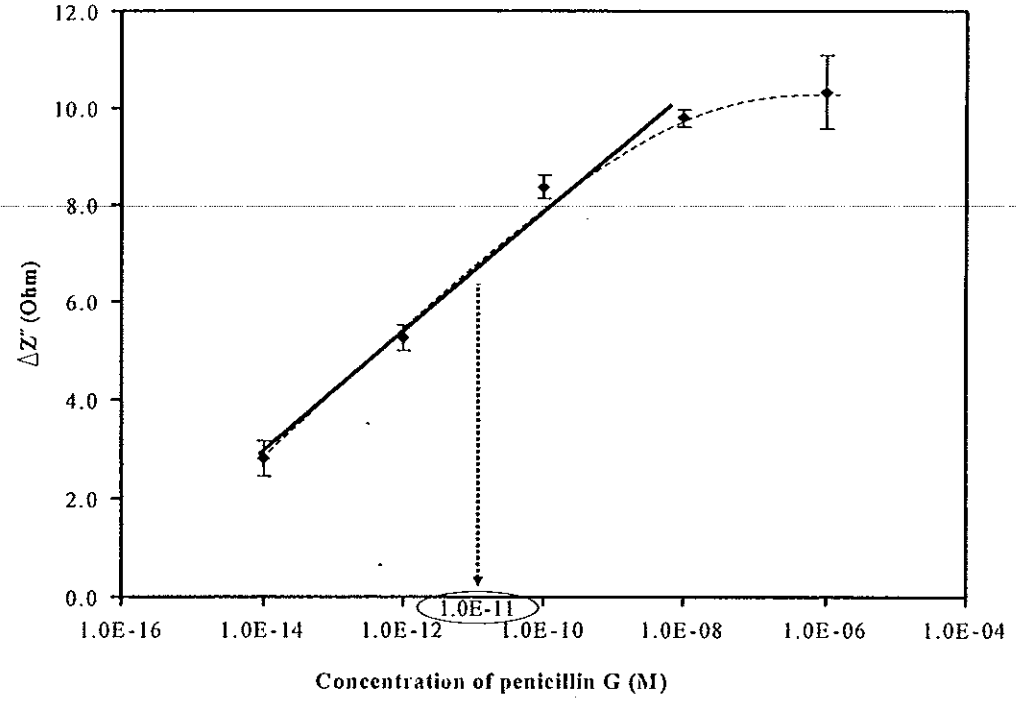


Figure 24 Responses of a flow injection impedimetric biosensor system for the primary test between penicillin G with anti-Penicillin G binding.

3.3.1 Flow rate

In a flow injection impedimetric biosensor system, the flow rate of the buffer passing through the flow cell is the main factor affecting the yield of interaction between penicillin G and immobilized anti-Penicillin G on the electrode surface. So optimization of flow rate is necessary. The changes in impedance obtained for different flow rates from 50 to 800 μl min^{-1} increased when the flow rate decreased (Table 5 and Figure 25) because at lower flow rate the analyte (penicillin G) had more time in the system to interaction with anti-Penicillin G than at higher flow rate.

The impedance change resulting from injections of 1×10^{-11} M of penicillin G standard gave the highest response at a flow rate of 50 μl min^{-1}. However, for flow rates between 50-200 μl min^{-1} the impedance changes were nearly the same, but the analysis time at 200 μl min^{-1} was much shorter. So the flow rate of 200 μl min^{-1} was chosen.

Table 5 Responses of a flow injection impedimetric biosensor system for penicillin G at different flow rates.

Flow rate ($\mu l\ min^{-1}$)	$\Delta Z''$ (Ohm) mean ± SD*	Analysis time** (min)
50	19.3 ± 0.6	40-45
100	18.7 ± 0.6	30-35
150	18.3 ± 0.6	20-25
200	17.7 ± 0.6	10-15
250	16.0 ± 1.0	7-12
300	14.7 ± 0.6	6-8
400	10.3 ± 0.6	6-8
500	8.7 ± 1.2	3-5
600	8.7 ± 0.6	3-5

*3 replications

**the time taken from injection until the response was stable

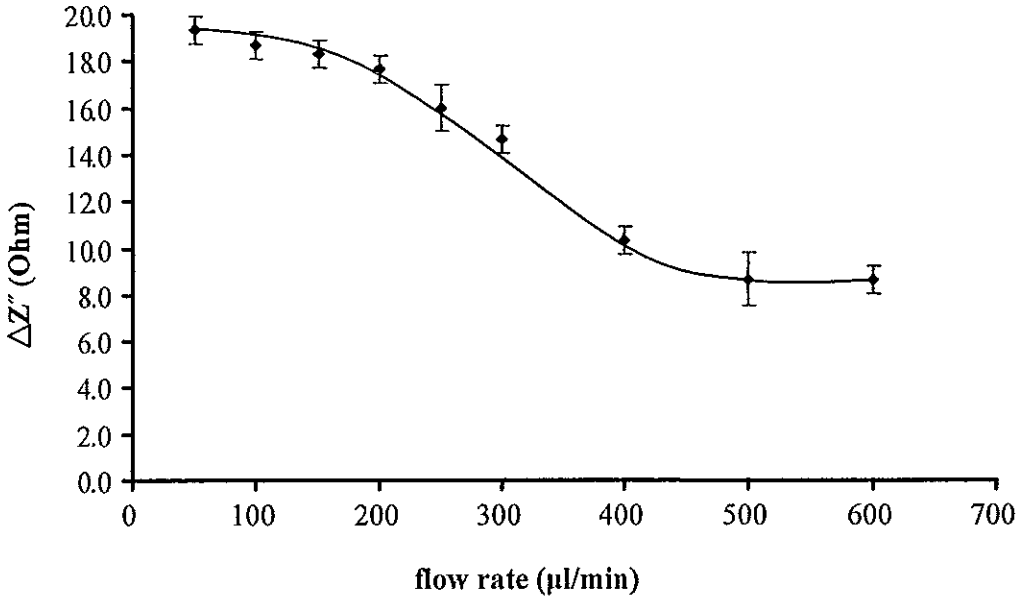


Figure 25 Effect of flow rates.

3.3.2 Type of buffer solution

The type of buffer used in a flow injection impedimetric immunosensor system, 10 mM phosphate buffer saline (Maupas *et al.*, 1997) and tris-buffered (Wang *et al.*, 2005; Wang *et al.*, 2006), were investigated (Table 6 and Figure 26) and 10 mM phosphate buffer pH 7.40 was chosen to be used in further analysis because it gave higher sensitivity and lower background.

Table 6 Responses of a flow injection impedimetric biosensor system for penicillin G at different type of buffer solution.

Concentration of penicillin G (M)	ΔZ" (Ohm) at different type of buffer	
	Phosphate buffer mean ± SD*	Tri-HCl buffer mean ± SD*
1.0×10^{-16}	5.7 ± 1.2	15.0 ± 1.4
1.0×10^{-14}	8.7 ± 0.6	34.0 ± 4.4
1.0×10^{-12}	14.0 ± 1.0	39.0 ± 4.0
1.0×10^{-10}	19.3 ± 1.2	46.3 ± 0.6
1.0×10^{-8}	26.0 ± 1.0	54.0 ± 1.0
Sensitivity (Ohm/log M)	2.87	2.40
r	0.998	0.984

*3 replications

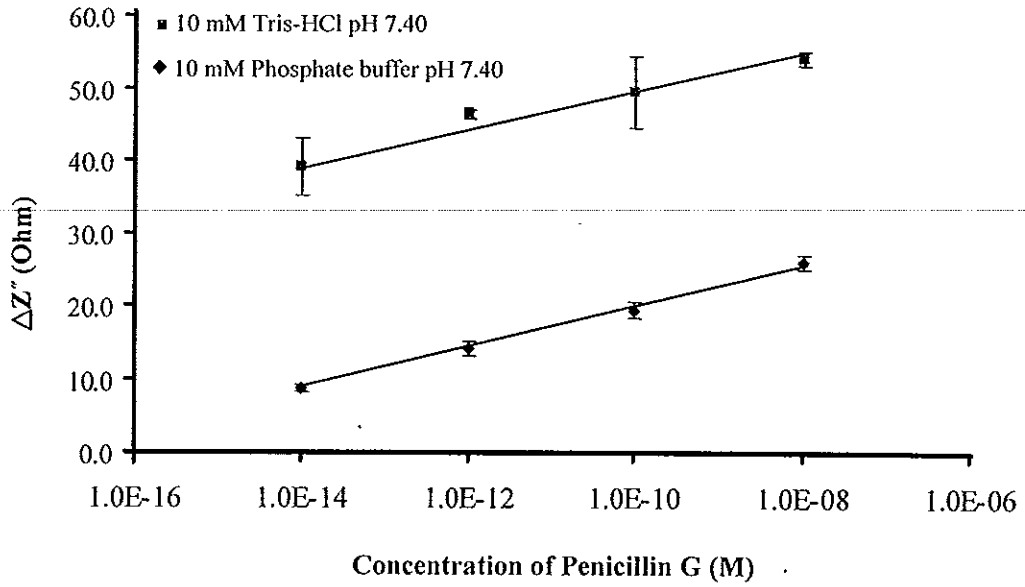


Figure 26 Effect of buffer solution.

3.3.3 pH of buffer solution

The influence of pH during binding reaction was studied using 10 mM phosphate buffer pH 6.80 to 7.60. The change of impedance (ΔZ") increased when increasing pH from 6.80 to 7.40 and decreased at pH 7.60 (Table 7 and Figure 27). The results show that the maximum change in impedance occurs at pH 7.40, i.e., at this pH the binding between penicillin G and anti-Penicillin G was the highest. This may be explained as follows. One of the binding forces of this affinity pair is electrostatic (Boehm *et al.*, 2000), therefore, it depends on the charges on the antibody and antigen. The isoelectric point of penicillin G is 2.3 (DrugBank, Canada) and the isoelectric point of anti-Penicillin G 6.1 – 8.5 (USBiological, USA). Therefore, in pH 7.40 penicillin G had the negative charge and anti-Penicillin G had positive charge and this help with the binding. It is possible that at this pH the different charges an each side of the affinity pair enable maximum binding compare to other pH. In addition pH 7.40 was widely used to study interaction of antibiotic to its antibody for immunosensor biosensor technique (Sternesjö *et al.*, 1995; Gaudin *et al.*, 2001; Park *et al.*, 2004), so pH 7.40 was chosen.

Table 7 Responses of a flow injection impedimetric biosensor system for penicillin G at different pH of buffer solution.

pH of phosphate buffer	ΔZ" (Ohm) mean ± SD*
6.80	1.4 ± 0.2
7.00	2.2 ± 0.2
7.20	3.3 ± 0.3
7.40	4.3 ± 0.2
7.60	2.9 ± 0.3

*3 replications

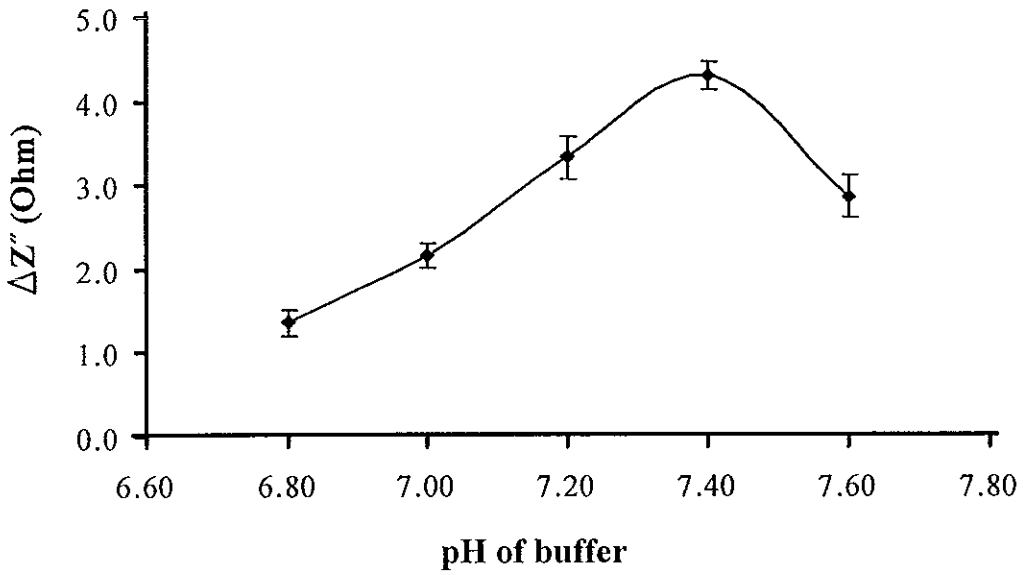


Figure 27 Effect of pH of buffer solution.

3.3.4 Sample volume

When different sample volumes were tested, impedance change increased with the sample volume from 50 to 200 µl (Table 8 and Figure 28) then became constant. Since the binding depends on the amount of immobilized antibody

on the electrode and equilibrium of antigen-antibody reaction if the analyte (antigen) exceeds the equilibrium the binding would not increase. Since the responses from 200-300 μl were nearly the same, 200 μl was chosen because it has the lowest analysis time.

Table 8 Responses of a flow injection impedimetric biosensor system for penicillin G at different sample volume.

Sample volume (μl)	ΔZ" (Ohm) mean ± SD*	Analysis time** (min)
50	4.7 ± 1.2	4 – 5
100	7.7 ± 0.6	5 – 7
150	9.0 ± 1.0	6 – 8
175	10.3 ± 0.6	7 – 8
200	12.3 ± 0.6	8 – 10
250	12.6 ± 0.6	12 – 15
300	12.3 ± 0.6	16 – 18

*3 replications

**the time taken from injection until the response was stable

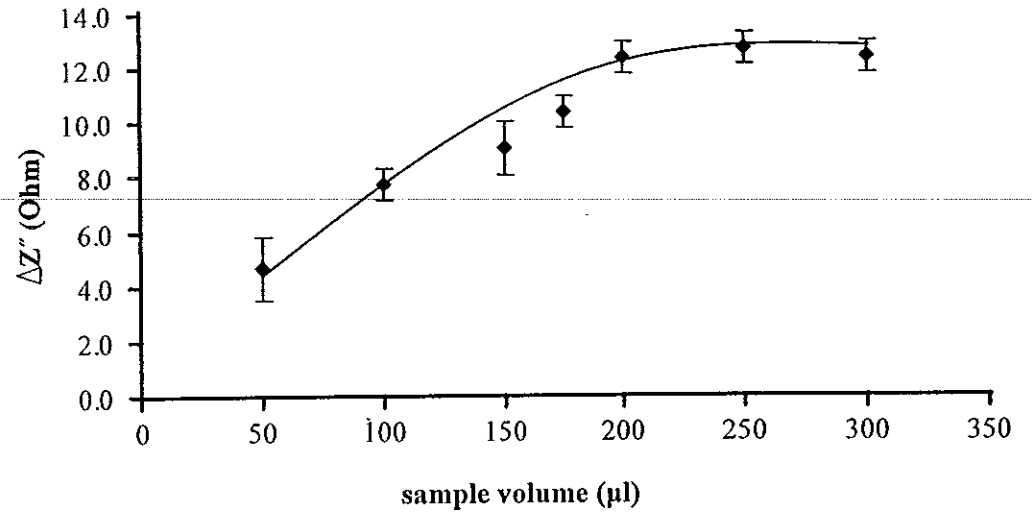


Figure 28 Effect of sample volume.

3.3.5 Type of regeneration solution

During each analysis penicillin G binds to anti-Penicillin G on the electrode surface causing the impedance to increase. After each analysis the analyte (penicillin G) needs to be removed so it can be used again for the next analysis. This was done by passing a volume of regeneration solution. The evaluation of the performance of the regeneration solution was done by considering the residual activity of the anti-Penicillin G electrode. This was calculated from the impedance change ($\Delta Z''$) resulting from the binding between penicillin G (1.0×10^{-11} M of penicillin G standard) and anti-Penicillin G before ($\Delta Z''_1$) and after regeneration ($\Delta Z''_2$) as follows;

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

The criteria for regenerating the electrode surface is "if post-regeneration binding remain above 90 % compared to the binding efficiency before regeneration, the used conditions should be seen as adequate" (Andersson *et al.*, 1999; van der Merwe, 2000).

Three types of regeneration solution were studied (Table 9, Figure 29);

(i) high ionic strength; 1.0 M NaCl, 1.0 M KCl, 1.0 M $MgCl_2$,

(ii) low pH; 0.1 M glycine-HCl pH 2.50 in 0.4 M NaCl, 0.1 M glycine-HCl pH 2.50, HCl pH 2.5 and

(iii) high pH; 0.005 M NaOH and 0.05 M NaOH.

In this study the volume of regeneration solution was the same as the volume of the analyte (penicillin G), 200 µl, because in a flow injection system both of these solutions were injected in the same line from the same sample loop.

The use of high pH as regeneration solutions were shown to be ineffective as the impedance signal (6.0 ± 1.0 and 5.3 ± 1.2) and percent residual activity were the lowest (48 ± 11 and 55 ± 9 %). For high ionic strength and low pH, they gave nearly the same impedance signal (7.3 ± 0.6 to 10.7 ± 0.6) and percent residual activity (67 ± 5 to 97 ± 5 %), however, high ionic strength solution required a long regeneration time (the time after injected regeneration solution to the baseline stable). Therefore 100 mM glycine-HCl pH 2.50 was chosen because it gave the highest impedance signal (10.7 ± 0.6), percent residual activity (97 ± 5%) and short analysis time (8 -12 min).

Table 9 Responses of a flow injection impedimetric biosensor system for penicillin G at different type of regeneration solution ($\Delta Z''_1$ = 11.0 Ohm).

Type of regeneration solution	$\Delta Z''_2$ (Ohm) mean ± SD*	% Residual activity mean ± SD*	Regeneration time** (min)
High ionic strength			
- 1 M NaCl	9.7 ± 0.6	88 ± 5	18-25
- 1 M KCl	9.7 ± 0.6	88 ± 5	19-25
- 1 M MgCl	7.3 ± 0.6	67 ± 5	25-30
Low pH			
- 100 mM glycine-HCl + NaCl, pH 2.50	8.0 ± 1.0	73 ± 9	14-18
- 100 mM glycine-HCl, pH 2.50	10.7 ± 0.6	97 ± 5	8-12
- HCl, pH 2.50	10.0 ± 1.0	91 ± 9	7-12
High pH			
- 5 mM NaOH	6.0 ± 1.0	55 ± 9	17-25
- 50 mM NaOH	5.3 ± 1.2	48 ± 11	18-24

*3 replications

**the time after injected regeneration solution to the baseline stable

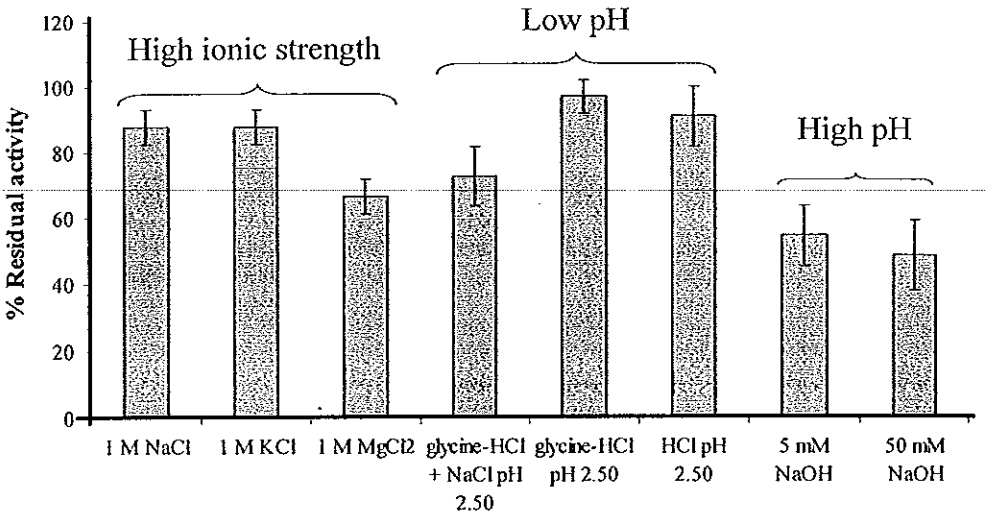


Figure 29 Effect of regeneration solution.

3.3.6 pH of regeneration solution

Using the best type of regeneration solution, glycine-HCl, the influence of pH ranging from 2.0 to 3.0 was studied (Table 10, Figure 30). The percent residual activity of anti-Penicillin G electrode increased as the pH decreased, i.e. lower pH can break the binding of penicillin G to anti-Penicillin G better than higher pH. High percent residual activity (93 ± 13 to 100 ± 11 %) were obtained at pH 2.60 down to 2.00, and pH 2.50 was chosen since it gave the lowest standard deviation and to avoid the possible deterioration of the insulating SAM layer as a result of low-pH (Jiang *et al.*, 2003).

Table 10 Responses of a flow injection impedimetric biosensor system for penicillin G at different pH of regeneration solution.

pH of glycine-HCl	ΔZ" (Ohm) mean ± SD*	% Residual activity mean ± SD*
2.00	9.0 ± 1.0	100 ± 11
2.10	8.7 ± 0.6	96 ± 6
2.20	8.3 ± 1.2	93 ± 13
2.30	8.3 ± 0.6	93 ± 6
2.40	8.7 ± 0.6	96 ± 6
2.50	9 ± 0	100 ± 0
2.60	8.3 ± 1.2	93 ± 13
2.70	6.7 ± 1.2	74 ± 13
2.80	7.6 ± 0.6	85 ± 6
2.90	7 ± 0	78 ± 0
3.00	7.0 ± 1.0	78 ± 11

*3 replications

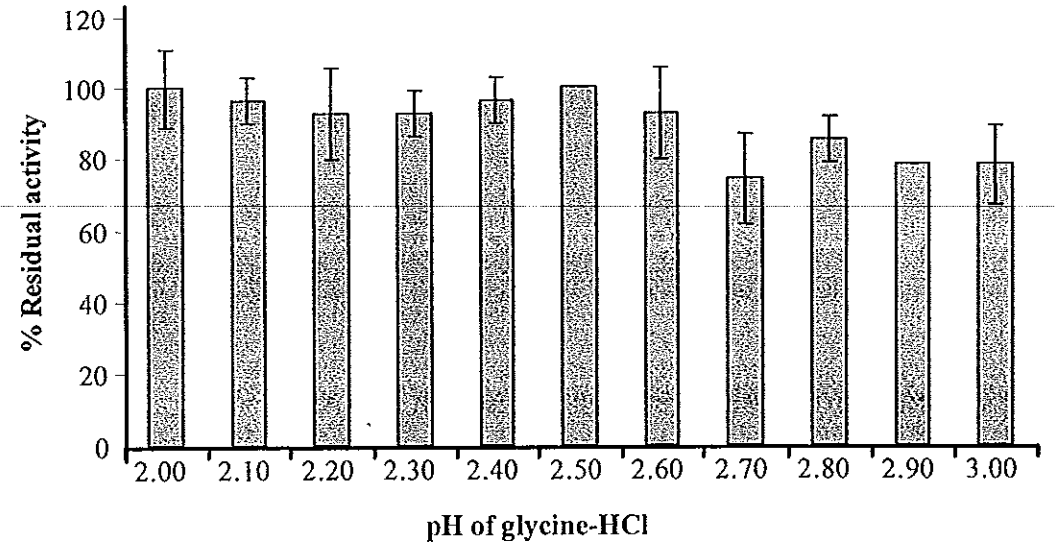


Figure 30 Effect of pH of regeneration solution.

3.3.7 Concentration of regeneration solution

The effect of concentration of glycine-HCl was studied (Table 11, Figure 31). At higher glycine-HCl concentrations (≥ 50 mM) the averages of percentage of residual activity values (100 ± 7 to 102 ± 4 %) above 90 % were achieved. Normally, if post-regeneration binding remains above 90 % compared to the binding efficiency before regeneration, the conditions should be seen as adequate. Therefore, any of these concentrations can be used. However, higher ionic strength solution may lead to production of pinholes on the electrode surface due to the removal of SAM (Jiang *et al.*, 2003). So, 50 mM glycine-HCl was chosen for concentration of regeneration solution.

Table 11 Responses of a flow injection impedimetric biosensor system for penicillin G at different concentration of regeneration solution, the regeneration time 10-12 min.

Concentration of glycine-HCl pH 2.50 (mM)	ΔZ" (Ohm) mean ± SD*	% Residual activity mean ± SD*
10	7.7 ± 0.6	55 ± 4
25	9.3 ± 0.6	67 ± 4
40	12.3 ± 0.6	88 ± 4
50	14.3 ± 0.6	102 ± 4
75	14.0 ± 1.0	100 ± 7
100	14.3 ± 0.6	102 ± 4

*3 replications

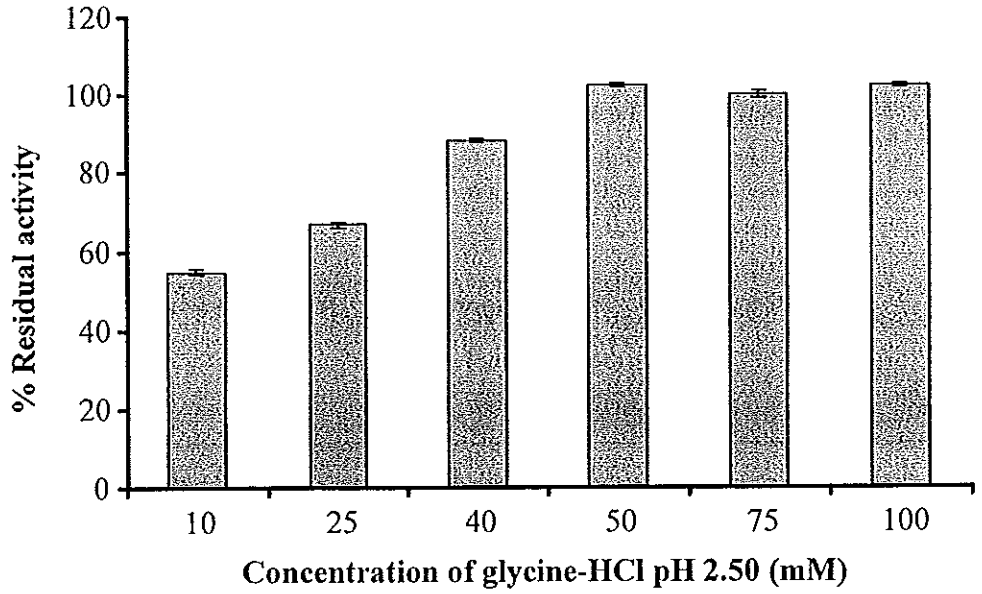


Figure 31 Effect of concentration of regeneration solution.

Optimum operating conditions are summarized in Table 12.

Table 12 Optimized operating conditions.

Conditions	Optimum values
Flow rate (μl min^{-1})	200
Buffer solution	10 mM phosphate buffer pH 7.40
Sample volume (μl)	200
Regeneration solution	50 mM glycine-HCl pH 2.50

3.4 Reproducibility

To test the reproducibility of the response penicillin G was analyzed 15 times per day for about 3-4 days under optimum conditions. After each analysis, 200 μl of 50 mM glycine-HCl buffer solution pH 2.50 was injected to break the binding between Penicillin G and anti-Penicillin G. The reproducibility of anti-Penicillin G modified electrode was evaluated by monitoring the impedance change ($\Delta Z''$) at the same concentration of standard Penicillin G (1.0×10^{-11} M) at a flow rate of 200 μl min^{-1}, 10 mM phosphate buffer solution, pH 7.40 and a sample volume of 200 μl.

Table 13 and Figure 32 show the percentage of residual activity of anti-Penicillin G electrode versus the number of injection. After 3 days or 45 times of regeneration the binding activity of anti-Penicillin G electrode retained about 95% of the original impedance change. This indicated that anti-Penicillin G electrode can be reused with good reproducibility up to 45 times with a relative standard deviation (RSD) of 3.85 %. The reduction of the activity may cause by the lost of anti-Penicillin G activity after prolonged used, removal of the antibody from the surface, or the lost of SAM layer.

Voltammetry was used to test these hypotheses and the results are shown in Figure 33. Curve a is the response when pinholes on the electrode surface were blocked by treating with 1-dodecanethiol before use and curve b is the response after reused for more than 50 times. The signal of curve a and b are nearly the same so SAM on gold electrode did not deteriorate. Therefore, the decrease of percent residual activity was most likely due to the lost of the antibody and/or its activity.

Table 13 Reproducibility of the response from the anti-Penicillin G modified electrode to injections of a fixed volume of a standard solution of penicillin G (1.0×10^{-11}M) with regeneration and reconditioning steps between each individual assay.

Number of injection	ΔZ" (Ohm)	% Residual activity	Number of injection	ΔZ" (Ohm)	% Residual activity
1	19	100	27	20	105
2	19	100	28	19	100
3	19	100	29	18	95
4	20	105	30	20	105
5	20	105	31	20	105
6	19	100	32	19	100
7	19	100	33	18	95
8	19	100	34	18	95
9	19	100	35	19	100
10	18	95	36	18	95
11	19	100	37	19	100
12	19	100	38	18	95
13	20	105	39	18	95
14	18	95	40	18	95
15	19	100	41	18	95
16	19	100	42	18	95
17	19	100	43	18	95
18	20	105	44	19	100
19	18	95	45	18	95
20	18	95	46	16	84
21	20	105	47	14	74
22	20	105	48	15	79
23	19	100	49	13	68
24	19	100	50	15	79
25	19	100	51	15	79
26	19	100			

Number of injection 1-45, % RSD = 3.85

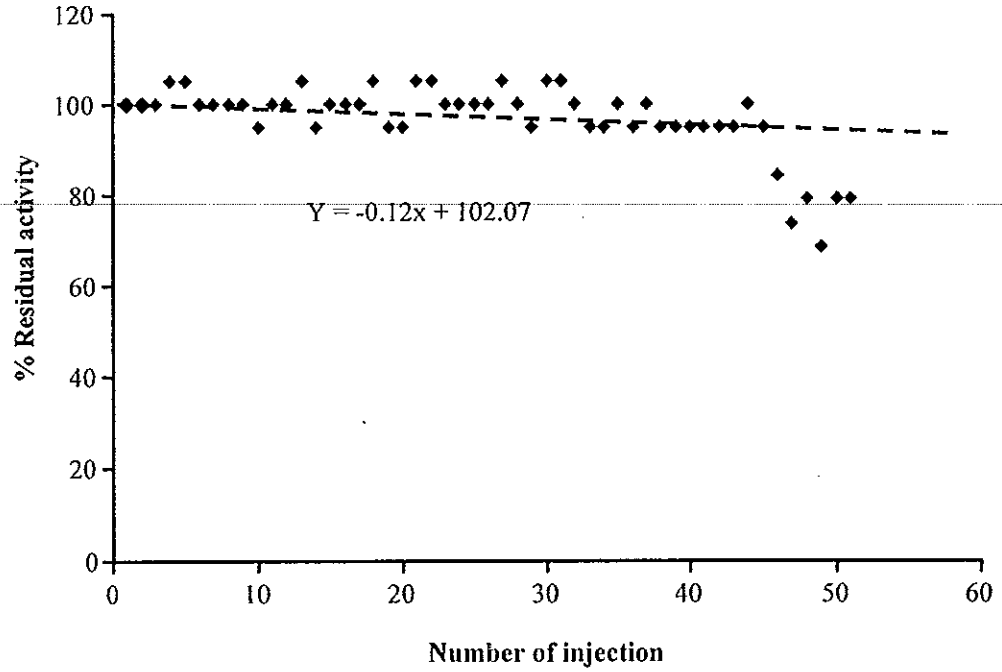


Figure 32 Reproducibility of the response from the anti-Penicillin G modified electrode to injections of a fixed volume of a standard solution of penicillin G (1.0×10^{-11}M) with regeneration and reconditioning steps between each individual assay.

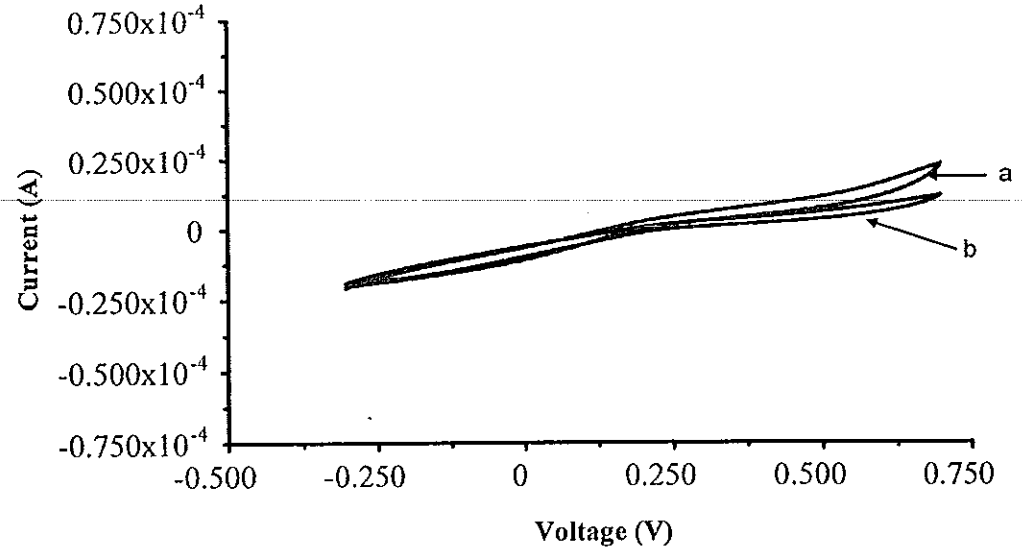


Figure 33 Cyclic voltammograms of gold electrode obtained in 0.05 M $K_3[Fe(CN)_6]$ with 0.1 M KCl, (a) is the response when pinholes on the electrode surface were blocked by 1-dodecanethiol before use and (b) is the response after reused for more than 50 times.

3.5 Linear dynamic range and detection limit

To investigate the linear dynamic range discrete pulse injection of penicillin G standard ranging from $1.0x10^{-18}$ to $1.0x10^{-6}$ M with intermediate regeneration steps using 50 mM glycine-HCl pH 2.50 were performed. Table 14 and Figure 34 show the calibration curve of 200 µl injections into 10 mM phosphate buffer solution, pH 7.40 at a flow rate of 200 µl min^{-1}. A linear relationship between the impedance change and logarithm of penicillin G concentration were obtained between $1.0x10^{-13}$ to $1.0x10^{-8}$ M. The detection limit was $3.0x10^{-15}$ M, based on IUPAC Recommendation 1994 (Buck and Lindner, 1994). The LOD was very much lower than other reports, i.e. 1.2×10^{-8} M reported by Gaudin *et al.* (2001) and 3.6×10^{-9} M reported by Benito-Peña *et al.* (2005). Therefore, the impedimetric immunosensor is suitable for quantitative analysis of penicillin G in milk since it gave the limit of detection (3.0×10^{-15} M) lower than the MRL (1.2×10^{-8} M).

Table 14 Responses of the impedance biosensor system under optimum conditions (200 μl min^{-1} flow rate, 200 μl sample volume, 10 mM phosphate buffer, pH 7.40).

Concentration of penicillin G (M)	ΔZ" (Ohm), mean ± SD*
1.0×10^{-18}	0.3± 0.6
1.0×10^{-17}	0.3 ± 0.6
1.0×10^{-16}	0.3 ± 0.6
1.0×10^{-15}	1.3 ± 0.6
1.0×10^{-14}	2.3 ± 0.6
1.0×10^{-13}	3.7 ± 0.6
1.0×10^{-12}	5.6 ± 0.6
1.0×10^{-11}	7.3 ± 0.6
1.0×10^{-10}	10.0 ± 0.6
1.0×10^{-09}	11.7 ± 1.2
1.0×10^{-08}	13.6 ± 0.6
1.0×10^{-07}	13.3 ± 0.6
1.0×10^{-06}	14.0 ± 1.0
Sensitivity (Ohm/log M) of penicillin G, 1.0×10^{-13}-1.0×10^{-08} M	2.020
r	0.998

Blank 0.5 ± 0.3* Ohm

*3 replications

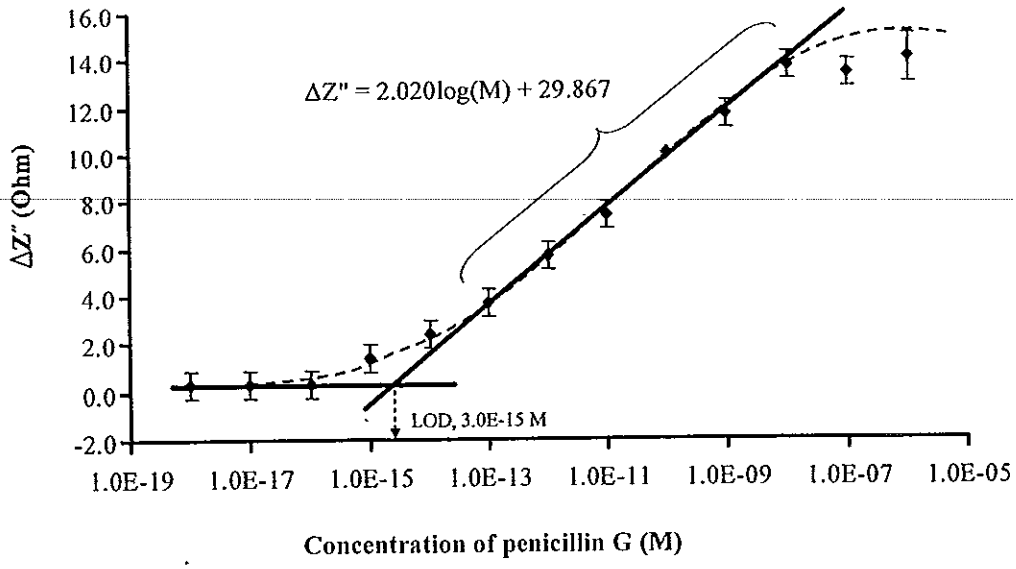


Figure 34 Impedance change vs. the logarithm of penicillin G concentration with immobilized anti-Penicillin G under the optimum conditions (200 µl min^{-1} flow rate, 200 µl sample volume, 10 mM phosphate buffer, pH 7.40).

3.6 Selectivity

The effect of substances that might interfere with the response of Penicillin G in the impedimetric biosensor system was studied. Ampicillin, amoxacillin trihydrate, cloxacillin, ciprofloxacin HCl and ofloxacin which have similar physical and chemical characteristics to penicillin G were used to test the selectivity of the impedimetric biosensor system.

Tables 15-17 and Figures 35-37 show the response of anti-Penicillin G to penicillin G, ampicillin, amoxacillin trihydrate, cloxacillin, ciprofloxacin HCl and ofloxacin at the same concentration range ($1.0x10^{-12}$ to $1.0x10^{-9}$ M). The impedance changes due to ampicillin, amoxacillin trihydrate, cloxacillin, ciprofloxacin HCl and ofloxacin were much lower than those from penicillin G. This system is much more selective than the one reported by Guadin *et al.* (2001), using antibody with high affinity to beta-lactams ring, i.e. the antibody can bind to all antibiotics in the group of beta-lactam. In this work monoclonal anti-Penicillin G was used so it reacted

specifically with penicillin G, not the beta-lactam ring (USBiological, USA). Since other antibiotics gave very lower response, the same response as blank which is generally the same or lower than the response at the limit detection of penicillin G, so their presence would not interfere with the detection of penicillin G.

Table 15 Responses of the anti-Penicillin G to penicillin G, amoxicillin, ampicillin and blank (running buffer).

Concentration (M)	ΔZ" (Ohm), mean ± SD*		
	Penicillin G	Amoxicillin	Ampicillin
1.0×10^{-12}	4.3 ± 0.6	0.7 ± 0.6	0.3 ± 0.6
1.0×10^{-11}	7.7 ± 0.6	0.7 ± 0.6	0.3 ± 0.6
1.0×10^{-10}	10.7 ± 06	0.3 ± 0.6	0.7 ± 0.6
1.0×10^{-09}	14.3 ± 0.6	0.3 ± 0.6	0.7 ± 0.6

Blank 0.7 ± 0.6* Ohm

*3 replications

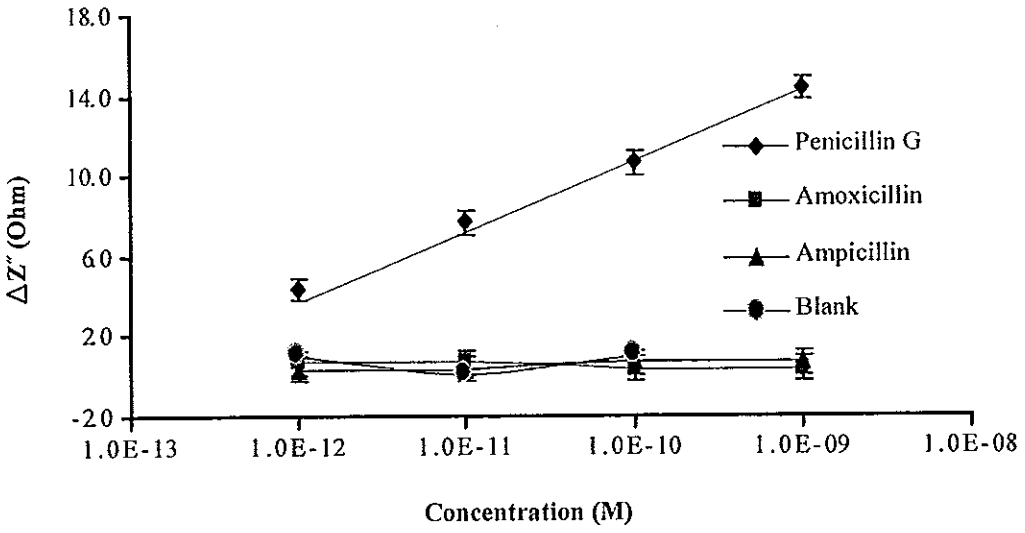


Figure 35 Effect of the anti-Penicillin G to penicillin G, amoxicillin, ampicillin and blank (running buffer).

Table 16 Responses of the anti-Penicillin G to penicillin G, ciprofloxacin HCl, ofloxacin and blank (running buffer).

Concentration (M)	ΔZ" (Ohm), mean ± SD*		
	Penicillin G	Ciprofloxacin HCl	Ofloxacin
1.0×10^{-12}	4.6 ± 0.6	0.3 ± 0.6	0.6 ± 0.6
1.0×10^{-11}	7.7 ± 0.6	0.3 ± 0.6	1.0 ± 0.0
1.0×10^{-10}	10.7 ± 06	0	0.3 ± 0.6
1.0×10^{-09}	13.0 ± 0.0	0.3 ± 0.6	1.0 ± 0.0

Blank 0.7 ± 0.6* Ohm

*3 replications

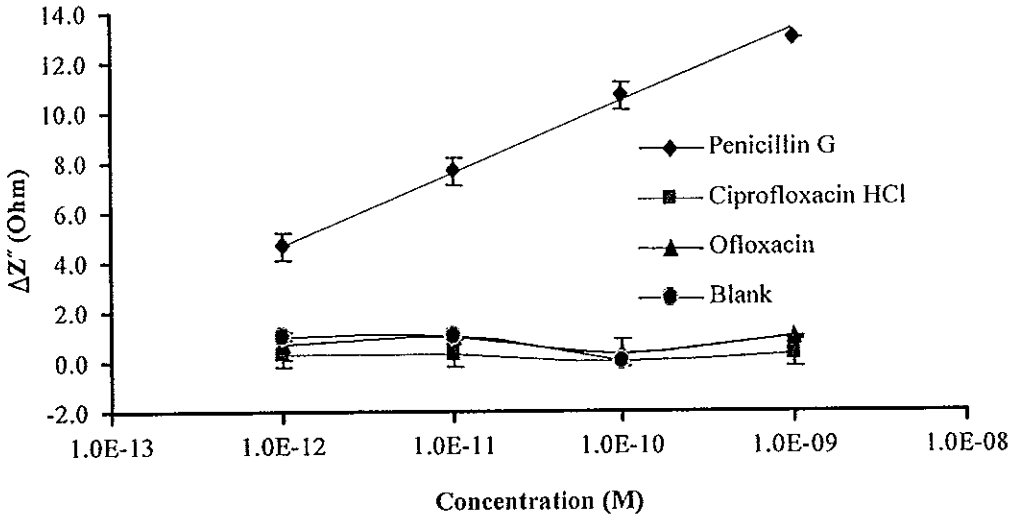


Figure 36 Effect of the anti-Penicillin G to penicillin G, ciprofloxacin HCl, ofloxacin and blank (running buffer).

Table 17 Responses of the anti-Penicillin G to penicillin G, cloxacillin and blank (running buffer).

Concentration (M)	ΔZ" (Ohm), mean ± SD*	
	Penicillin G	Cloxacillin
1.0×10^{-12}	3.8 ± 0.2	0.5 ± 0.3
1.0×10^{-11}	5.8 ± 0.2	0.7 ± 0.2
1.0×10^{-10}	8.7 ± 0.2	0.7 ± 0.2
1.0×10^{-09}	10.7 ± 0.1	0.6 ± 0.3

Blank 0.8 ± 0.1* Ohm

*3 replications

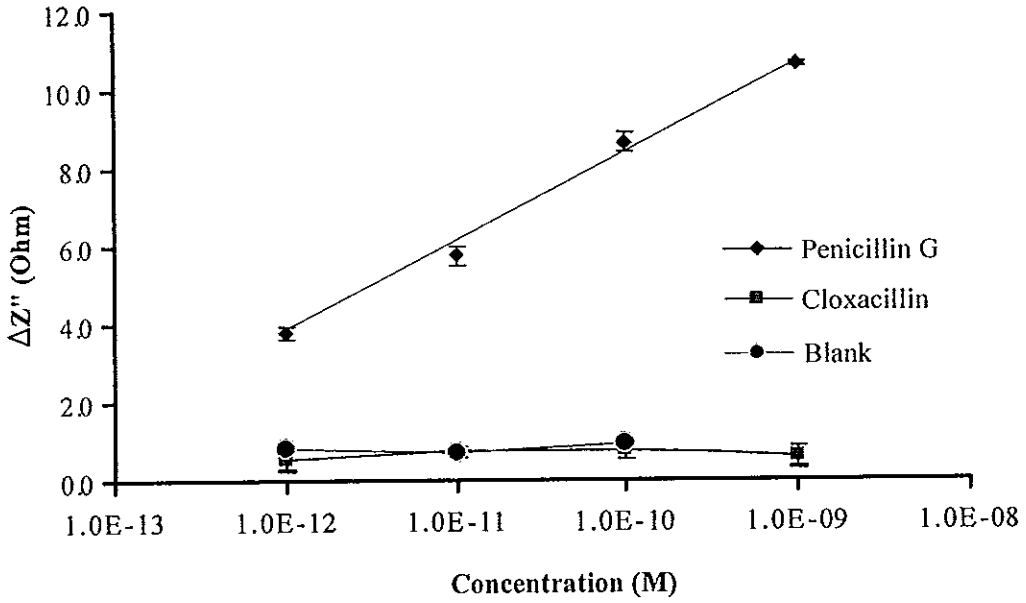


Figure 37 Effect of the anti-Penicillin G to penicillin G, cloxacillin and blank (running buffer).

3.7 Treatment of real samples

To demonstrate the use of the impedimetric immunosensor, raw milk samples obtained from Department of Animal Science, Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Thailand and pasteurized milk (no fat and low fat (5% fat)) from the store were tested. Two procedure were investigates.

3.7.1 Centrifugation plus dilution

Centrifugation was used to defat (Gaudin *et al.*, 2001; Gustavsson *et al.*, 2002) and, thus, reduce the matrix in milk sample. In this method, raw milk samples were centrifuged (Spectrafuge 16 M, Labnet, USA) at 14,000 rpm for 15 min. The aqueous phase was then recentrifuged under the same condition. The aqueous phase of this second centrifugation was filtered with Alber® nylon membrane (Albet, Spain pore size 0.22 μm) and then diluted for 1,000 (sample 103 and 113) and 10,000 times (sample 373, 403 and 418) using 10 mM phosphate buffer pH 7.40. The samples were injected into the impedimetric system and the responses were use to calculate penicillin G concentration from the calibration curve done prior to the test (Table 18, Figure 38).

Table 19 and Figure 39 show the concentration of penicillin G in raw milk where high standard deviations were obtained. This may be because the calibration curve was done on a log scale and hence a slight change in response would provide quite a different concentration values (Table 18 and Figure 38). Therefore, this calibration curve is not suitable for quantitative analysis of penicillin G. A calibration curve between the impedance change and concentration of penicillin G standard would provide a more accurate result.

Table 18 Calibration curves using determine penicillin G in raw milk by centrifuge then dilution, plotting the impedance change (ΔZ″) *vs.* logarithm of penicillin G concentration (M).

Concentration of penicillin G (M)	ΔZ" (Ohm), mean ± SD*
1.0×10^{-11}	5.0 ± 0.0
1.0×10^{-10}	9.7 ± 0.6
1.0×10^{-09}	14.7 ± 0.6
1.0×10^{-08}	18.3 ± 0.6
Sensitivity (Ohm/log M)	4.501
r	0.998

*3 replications

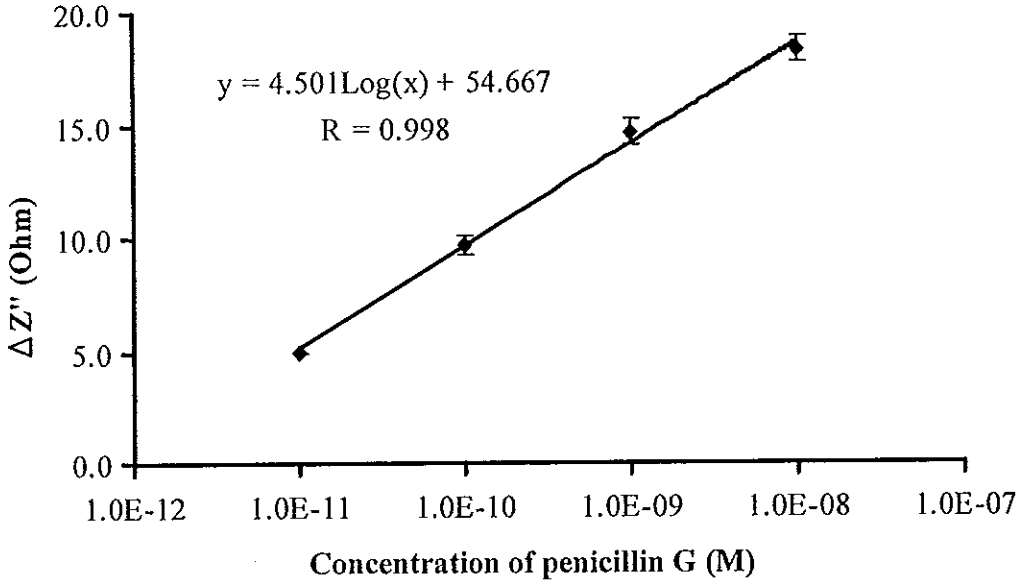


Figure 38 Calibration curves using determine penicillin G in raw milk by centrifuge then dilution, plotting the impedance change (ΔZ″) *vs.* logarithm of penicillin G concentration (M).

Table 19 The concentration of penicillin G in raw milk by centrifuge the sample then dilution.

Sample No.	Concentration of penicillin G in raw milk (10^{-7} M), mean ± SD*
103	67 ± 21
113	111 ± 35
373	40 ± 13
403	47 ± 13
418	10 ± 3

*3 replications

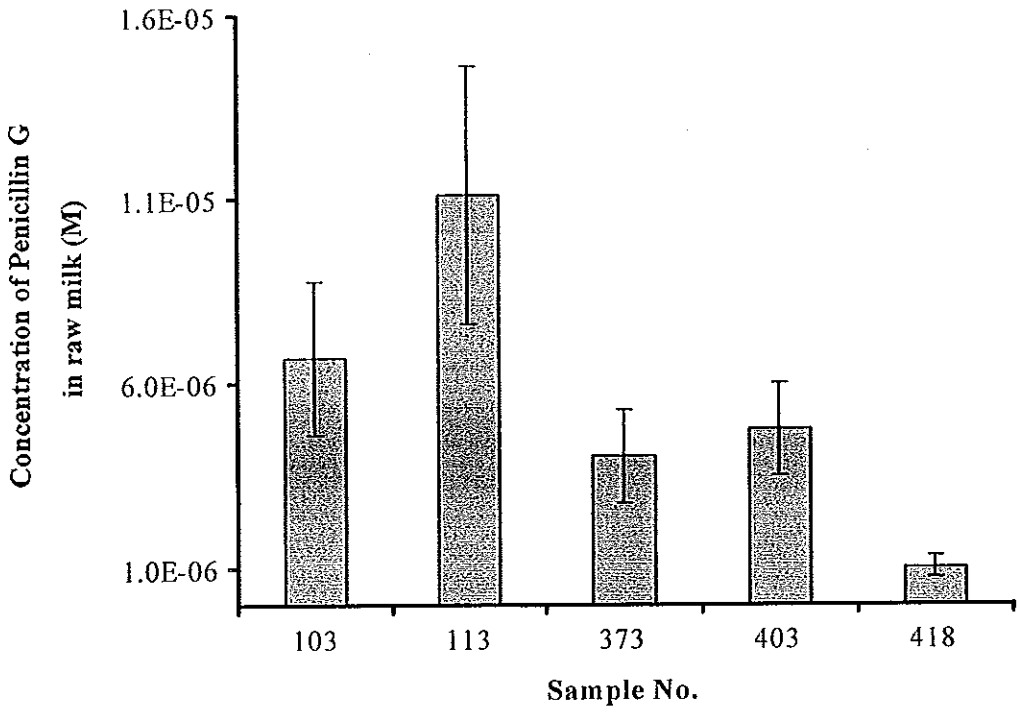


Figure 39 The concentration of penicillin G in raw milk by centrifuge the sample then dilution.

3.7.2 Dilution

In previous work centrifugation was applied, however, it was rather time consuming. Since in this system the LOD is very low, 3.0×10^{-15} M, and the MRL is only 1.2×10^{-8} M, therefore, dilution of several orders can be applied to reduce the matrix effect while the system is still able to detect the analyte.

In this procedure, pasteurized milk samples (no fat and low fat) were spiked with standard penicillin G at 1.0×10^{-8} M, the same concentration as the MRL. They were diluted 10 and 100 times using 10 mM phosphate buffer pH 7.40. These dilutions were tested and the analysis times were 30-40 min and 90-115 min for 100x and 10x dilution, respectively (Table 20). The longer analysis time of the 10x dilution could be due to the adsorption of milk matrix in the system and needed longer washing time. Therefore, more dilution was needed.

A calibration of 10,000 times was then tested. It provided short analysis time (10 – 12 min) and regeneration time (10 - 12 min), i.e. 20 - 24 min is required for one analysis, the same analysis times as standard penicillin G and this was used in the remaining test.

Table 20 The response of spiked no fat and low fat pasteurized milk (penicillin G 1.0×10^{-8} M) at different dilution.

Sample	ΔZ'' (Ohm) mean ± SD*	Analysis time** (min)
10 dilution time		
- no fat	18.3 ± 0.6	90 – 100
- low fat	18.0 ± 1.0	105 - 115
100 dilution time		
- no fat	5.7 ± 0.6	30 – 35
- low fat	5.7 ± 0.6	35 – 40

*3 replications

**the time after inject analyte to the response stable

3.8 Validation of method

Method validation is done by evaluating recovery and precision of the method. The calibration curve of penicillin G was prepared under optimum conditions. Since a linear relationship between the impedance changes and logarithm of penicillin G concentration is suitable for an "order of magnitude" test of penicillin G. Therefore, to obtain a good quantitative analysis of penicillin G in milk samples, a calibration curve between the impedance changes and concentration of standard penicillin G was used because it would provide more accurate result.

3.8.1 Recovery

The recovery of penicillin G was tested by spiking raw milk samples with penicillin G at 1.0×10^{-9}, 5.0×10^{-9}, 1.0×10^{-8}, 5.0×10^{-8} and 1.0×10^{-7} M. With 10,000 times dilution the concentrations would be between 1.0×10^{-13} and 1.0×10^{-11} M. Therefore, calibration curve using standard solutions were done in this range. Two calibration curves of standard penicillin G are shown in Table 21 and Figure 40.

Table 21 The response of penicillin G standard solution (for sample 113).

Concentration of penicillin G (M)	ΔZ" (Ohm), mean ± SD*
1st calibration curve	
1.0×10^{-13}	2.8 ± 0.2
2.5×10^{-13}	4.5 ± 0.1
5.0×10^{-13}	6.0 ± 0.1
7.5×10^{-13}	7.5 ± 0.2
1.0×10^{-12}	9.1 ± 0.1
Sensitivity (Ohm/M)	6.727×10^{12}
r	0.995
2nd calibration curve	
2.5×10^{-12}	10.5 ± 0.4
5.0×10^{-12}	11.6 ± 0.4
7.5×10^{-12}	12.6 ± 0.3
1.0×10^{-11}	13.3 ± 0.3
Sensitivity (Ohm/M)	3.803×10^{11}
r	0.995

*3 replications

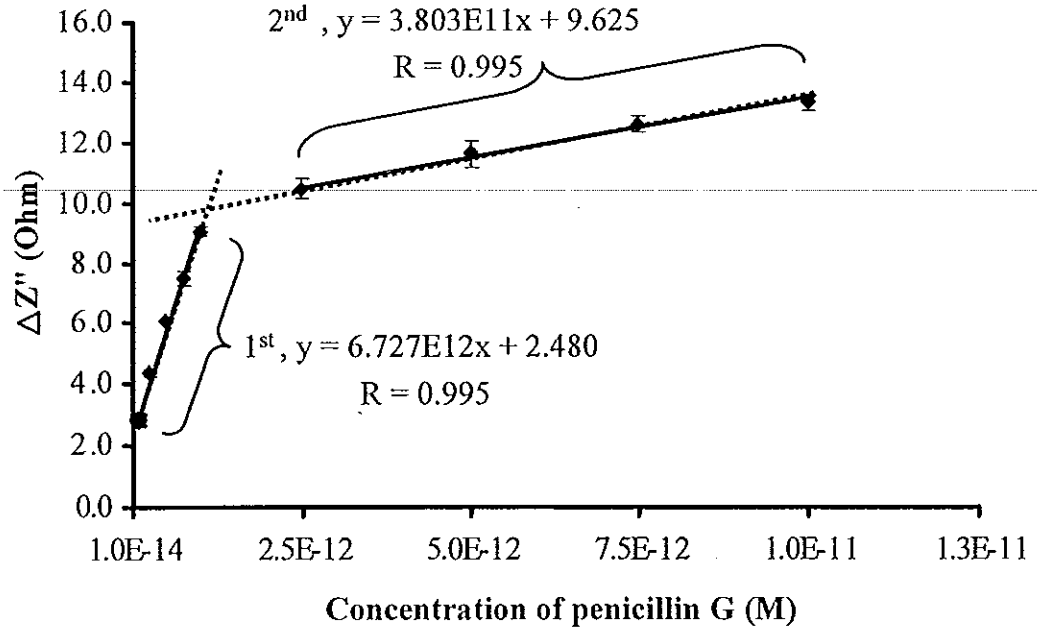


Figure 40 Calibration curve of penicillin G standard solution (for sample 113).

The spiked milk samples were diluted 10,000 times with 10 mM phosphate buffer pH 7.40, injected into the impedimetric biosensor system and the responses were obtained. Table 22 shows the response and percent recovery in raw milk (sample 113). From the values of the responses the concentrations were interpret accordingly. That is, when the response was between 2.6 to 9.8 ohm the first calibration curve was used, and when the response was between 9.8 to 13.6 ohm the second calibration curve was used. The concentration and percent recovery of order 1-4 were interpreted by the first calibration curve and order 5-6 used the second calibration curve.

Table 22 Percentage recovery of penicillin G spike raw milk sample 113 at spiked concentration $1.0x10^{-9}$, $5.0x10^{-9}$, $1.0x10^{-8}$, $5.0x10^{-8}$and $1.0x10^{-7}$ M and diluted 10,000 times.

Order	Concentration (x 10^{-13}M)		ΔZ" (Ohm)	Recovery (%)	
	Added	Found mean ± SD*	mean ± SD*	mean ± SD*	RSD*
1	0 (sample No. 113)	**ND	0.2 ± 0.2	-	-
2	1.0	0.8 ± 0.1	3.0 ± 0.1	76 ± 8	11
3	5.0	3.8 ± 0.1	5.0 ± 0.1	76 ± 2	2
4	10.0	8.5 ± 0.5	8.2 ± 0.4	85 ± 5	6
5	50.0	41 ± 3	11.2 ± 0.1	81 ± 6	8
6	100.0	84 ± 3	12.8 ± 0.1	84 ± 3	3

*3 replications

**ND = non detectable

Since an electrode can be reused up to 45 times (see 3.4) to repeat the recovery test in a new sample a new electrode was prepared and new standard calibration curves were prepared. Two more samples were tested (sample 373 and 403). For sample 373 the calibration curves are shown in Table 23 and Figure 41 and the results of percent recovery in Table 24. For sample 403, the calibration curves are shown in Table 25 and Figure 42 and the results of percent recovery are in Table 26.

Table 23 The response of penicillin G standard solution (for sample 373).

Concentration of penicillin G (M)	ΔZ" (Ohm), mean ± SD*
1st calibration curve	
1.0×10^{-13}	2.8 ± 0.2
2.5×10^{-13}	4.0 ± 0.2
5.0×10^{-13}	5.3 ± 0.2
7.5×10^{-13}	7.2 ± 0.2
1.0×10^{-12}	9.0 ± 0.2
Sensitivity (Ohm/M)	6.773×10^{12}
r	0.998
2nd calibration curve	
2.5×10^{-12}	10.0 ± 0.2
5.0×10^{-12}	11.5 ± 0.3
7.5×10^{-12}	12.6 ± 0.4
1.0×10^{-11}	13.7 ± 0.5
Sensitivity (Ohm/M)	4.905×10^{11}
r	0.997

*3 replications

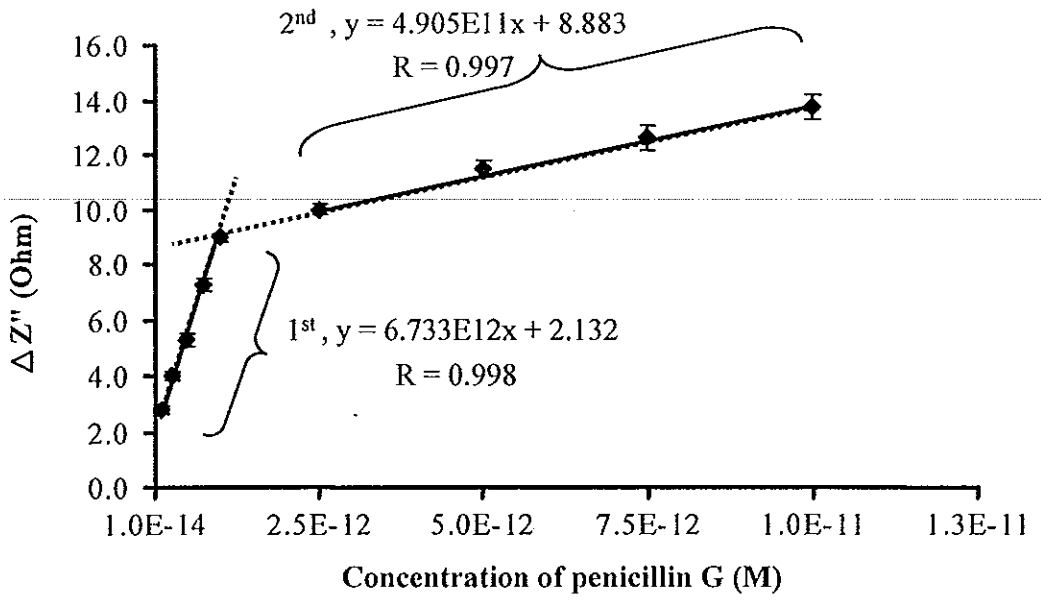


Figure 41 Calibration curve of penicillin G standard solution (for sample 373).

Table 24 Percentage recovery of penicillin G spike raw milk sample 373 at spiked concentration $1.0x10^{-9}$, $5.0x10^{-9}$, $1.0x10^{-8}$, $5.0x10^{-8}$and $1.0x10^{-7}$ M and diluted 10,000 times.

Order	Concentration (x 10^{-13}M)		ΔZ'' (Ohm)	Recovery (%)	
	Added	Found mean ± SD*	mean ± SD*	mean ± SD*	RSD*
1	0 (sample No. 373)	1.2 ± 0.5	2.9 ± 0.3	-	-
2	1.0	0.6 ± 0.2	3.3 ± 0.1	64 ± 15	24
3	5.0	3.7 ± 0.5	5.4 ± 0.3	75 ± 9	13
4	10.0	7.3 ± 0.5	7.9 ± 0.3	73 ± 5	7
5	50.0	38 ± 4	10.8 ± 0.2	76 ± 8	10
6	100.0	78 ± 8	12.8 ± 0.4	78 ± 8	10

*3 replications

Table 25 The response of penicillin G standard solution (for sample 403).

Concentration of penicillin G (M)	ΔZ" (Ohm), mean ± SD*
1st calibration curve	
1.0×10^{-13}	2.4 ± 0.3
2.5×10^{-13}	3.9 ± 0.2
5.0×10^{-13}	5.6 ± 0.3
7.5×10^{-13}	7.5 ± 0.4
1.0×10^{-12}	8.9 ± 0.4
Sensitivity (Ohm/M)	7.181×10^{12}
r	0.995
2nd calibration curve	
2.5×10^{-12}	10.5 ± 0.5
5.0×10^{-12}	11.4 ± 0.4
7.5×10^{-12}	12.2 ± 0.1
1.0×10^{-11}	12.8 ± 0.6
Sensitivity (Ohm/M)	3.134×10^{11}
r	0.995

*3 replications

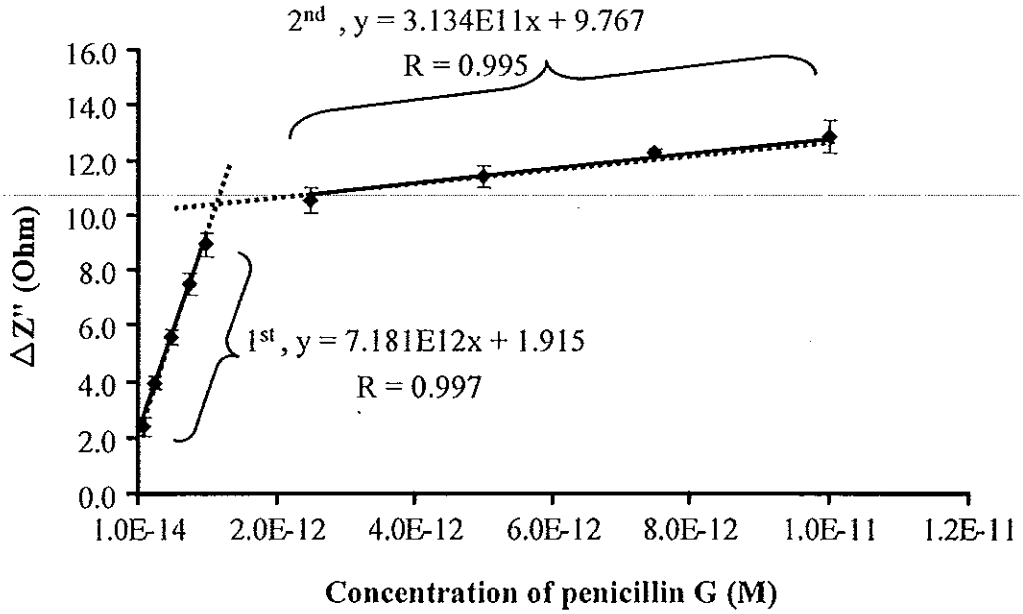


Figure 42 Calibration curve of penicillin G standard solution (for sample 403).

Table 26 Percentage recovery of penicillin G spike raw milk sample 403 at spiked concentration $1.0x10^{-9}$, $5.0x10^{-9}$, $1.0x10^{-8}$, $5.0x10^{-8}$and $1.0x10^{-7}$ M and diluted 10,000 times.

Order	Concentration (x 10^{-13}M)		ΔZ'' (Ohm)	Recovery (%)	
	Added	Found mean ± SD*	mean ± SD*	mean ± SD*	RSD*
1	0 (sample No. 403)	1.0 ± 0.4	2.6 ± 0.2	-	-
2	1.0	1.0 ± 0.4	3.4 ± 0.2	101 ± 36	35
3	5.0	4.5 ± 0.5	5.9 ± 0.3	90 ± 9	10
4	10.0	8.2 ± 0.2	8.5 ± 0.2	82 ± 2	3
5	50.0	45 ± 6	11.2 ± 0.2	90 ± 13	14
6	100.0	84 ± 9	12.4 ± 0.3	84 ± 9	10

*3 replications

The acceptable values of percent recovery and RSD at different concentration range are presented in Tables 27 and 28 (Taverniers *et al.*, 2004). From the results percentages of recovery (64 ± 15 to 101± 36 %) and relative standard

deviation (3 – 35 %) of penicillin G spiked in three raw milk samples are acceptable for these concentration ranges (recovery is 40–120 %, RSD is 30–45.3 %).

Table 27 Percentages of recovery can acceptable as a function of the analyte concentration (Taverniers *et al.*, 2004).

Analyte %	Analyte ratio	Unit	Mean recovery (%)
100	1	100%	98-102
10	1.00E-01	10%	98-102
1	1.00E-02	1%	97-103
0.1	1.00E-03	0.01%	95-105
0.01	1.00E-04	100 ppm	90-107
0.001	1.00E-05	10 ppm	80-110
0.0001	1.00E-06	1 ppm	80-110
0.00001	1.00E-07	100 ppb	80-110
0.000001	1.00E-08	10 ppb	60-115
0.0000001	1.00E-09	1 ppb	40-120

Table 28 Percentages of relative standard deviation can acceptable as a function of the analyte concentration (Taverniers *et al.*, 2004).

Analyte %	Analyte ratio	Unit	Horwiz %RSD	AOAC PVM %RSD
100	1	100%	2	1.3
10	1.00E-01	10%	2.8	2.8
1	1.00E-02	1%	4	2.7
0.1	1.00E-03	0.01%	5.7	3.7
0.01	1.00E-04	100 ppm	8	5.3
0.001	1.00E-05	10 ppm	11.3	7.3
0.0001	1.00E-06	1 ppm	16	11
0.00001	1.00E-07	100 ppb	22.6	15
0.000001	1.00E-08	10 ppb	32	21
0.0000001	1.00E-09	1 ppb	45.3	30

It can also be seen that the three different electrodes provided calibration curves with different sensitivities (Figures 40-42). Therefore, when changing to a new electrode recalibration is required.

3.8.2 Precision

The precision of a method is the degree of repeatability of analytical under normal operation and is normally expressed as the percent relative standard deviation for a statically significant number of samples. It is generally independent of recovery but it is a function of the concentration at which it was measured. Milk samples diluted with 10 mM phosphate buffer pH 7.40 and standard penicillin G were prepared in the same buffer and injected into the impedimetric biosensor system. Fives replicates were done for each sample and forty-five replicates for the standard penicillin G (Table 29).

Table 29 Precisions of raw milk samples and standard penicillin G (1.0×10^{-11} M).

Sample	ΔZ" (ohm)	
	mean ± SD	% RSD
103*	11.8 ± 0.5	3.8
113*	10.6 ± 0.4	3.6
418*	9.9 ± 0.3	2.6
standard penicillin G (1.0×10^{-11} M)**	18.9 ± 0.8	3.9

*5 replications
**45 replications

The results show good relative standard deviations (< 4%) of the same concentration and sample. Impedimetric immunosensor provides quantitative penicillin G in milk sample.

CHAPTER 4

CONCLUSIONS

Flow injection impedimetric immunosensor has been developed to determine penicillin G in milk samples based on the immobilization of anti-Penicillin G on self-assembled thioctic acid monolayer (SAM) via covalent coupling on gold working electrode. Binding of penicillin G to anti-Penicillin G on the working electrode causes the impedance to increase. Real time monitoring of the impedance was carried out at 160 Hz, the optimum frequency.

Optimum operating conditions of a flow impedimetric system were investigated and found to be as shown in Table 30

Table 30 optimum conditions of flow injection impedimetric immunosensor

Condition	Optimum values
Flow rate	200 µl min^{-1}
Type of buffer	10 mM phosphate buffer
pH of buffer	7.40
Sample volume	200 µl
Type of regeneration	glycine-HCl
pH of regeneration	2.50
Concentration of regeneration	50 mM

At optimum conditions good performance was obtained. The impedimetric immunosensor system provided a linear range between 1.0×10^{-13} to 1.0×10^{-8} with a detection limit of 3.0×10^{-15} M, analysis time 10-12 min and regeneration time 10-12 min. The system is specific for penicillin G and can be regenerated and reused up to 45 times.

This technique was validated to ensure the reliability of the results by using standard, milk samples and spike milk samples. Validation parameters, percentage of recovery and precision, were studied. Percentage recoveries were

obtained in acceptable level (Taverniers *et al.*, 2004), ranged from 64-101 at spiked concentration 1.0 x 10^{-13} to 1.0 x 10^{-11} M from two calibration curves. Good precision was also obtained with relative standard deviations (RSD) in raw milk samples and standard penicillin G (1.0 x 10^{-11} M) lower than 4 %.

Tables 31-35 show the comparison between the analytical features of the flow injection impedimetric immunosensor, chromatography, microbiology and commercial test kit and the other biosensor systems. It can be seen that these flow injection impedimetric immunosensor provide much better performances than other methods in almost all categories. These results show that the flow injection impedimetric immunosensor can use to determine penicillin G in milk samples. This technique is highly selective, can detect analyte with accuracy, using short analysis time (10-12 min) and regeneration time (10-12 min), low detection limit (3.0 x 10^{-15} M) lower than the MRL (1.2 x 10^{-8} M). The preparation of modified electrode although requires several steps, is quite simple. The regeneration of activity of the working electrode can be performed by glycine-HCl with good reproducibility (% RSD < 3.9) enable the electrode to reuse for many times.

Table 31 Comparison of the analytical features between chromatography and impedimetric immunosensor for penicillin G.

Analytical feature	LC-UV[1]	LC-UV[2] (derivatized)	LC-MS[3]	CE[4]	Impedimetric immunosensor
LOD (M)	2.6×10^{-8}	9.0×10^{-9}	6.0×10^{-9}	1.1×10^{-5}	3.0×10^{-15}
Linear range (M)	2.8×10^{-3} to 2.8×10^{-8}	1.2×10^{-7} to 1.2×10^{-8}	2.6×10^{-8} to 2.7×10^{-9}	2.8×10^{-3} to 4.2×10^{-5}	1.0×10^{-8} to 1.0×10^{-13}
Sample preparation					
- Extraction	Yes	Yes	Yes	Test only standard	No
- Clean-up	Yes	Yes	Yes	Test only standard	No

[1]LC-UV for penicillin G (Boatto *et al.*, 1998)

[2]LC-UV derivatized for penicillin G (Brito and Junqueira, 2006)

[3]LC-MS for penicillin G (Riediker *et al.*, 2001)

[4]CE for penicillin G (Pajchel *et al.*, 2004)

Table 32 Comparison of the analytical features between microbiology and impedimetric immunosensor for penicillin G.

Analytical feature	982.15*	982.18*	988.08*	Impedimetric immunosensor
LOD (ppb)	6	6	4.8	1.0×10^{-6}
Analysis time	> 30 min	3 h	> 30 min	10-12 min
Reusable	No	No	No	Yes

* American Organization of Analytical Chemistry (AOAC) method

Table 33 Comparison of the analytical features between commercial test kit and impedimetric immunosensor for penicillin G.

Analytical feature	Charm II® (*), (**)	Delvotest SP or Delvotest® (*), (**)	Penzyme® (***)	Impedimetric immunosensor
LOD (ppb)	2	3	5	1.0×10^{-6}
Analysis time	3 h	2.5 - 3 h	20 min	10-12 min
Cost	350 baht	100 baht ($2.66)	90 baht ($2.28)	40 baht****
Reusable	No	No	No	Yes

* Grunwald and Petz, 2003

**Popelka *et al.*, 2004

***Zeng *et al.*, 1996

**** Material cost

Table 34 Comparison of the analytical features between enzymatic system (potentiometric, amperometric and SPR) and impedimetric immunosensor for penicillin G.

Analytical feature	Potentiometric(1), (2)	Amperometric (3)	SPR (4)	Impedimetric immunosensor
LOD (M)	(1) 1.5×10^{-2} (2) 5.0×10^{-6}	2.0×10^{-6} to 1.0×10^{7}	3.6×10^{-9} , 4.5×10^{9}	3.0×10^{-15}
Linear range (M)	(1) 1.0×10^{-1} to 4.0×10^{-2} (2) 1.0×10^{-3} to 5.0×10^{-5}	2.0×10^{-3} to 4.0×10^{-6}	1.8×10^{-8} to 3.0×10^{-9}	1.0×10^{-8} to 1.0×10^{-13}

(1) Eppelsheim *et al.* (1998)

(2) Poghossian *et al.* (2001)

(3) Stred'anky *et al.* (2000)

(4) Gustavsson *et al.* (2004)

Table 35 Comparison of the analytical features between labeled, label-free and impedimetric immunosensor for penicillin G.

Analytical feature	Labeled(1), (2)	Labeled-free (SPR) (3)	Impedimetric immunosensor
LOD (M)	(1) 1.2×10^{-8} (2) 7.2×10^{-9}	1.2×10^{-8}	3.0×10^{-15}
Analysis method	(1) Screening (2) quantitative	screening	Quantitative
Sample pre-treatment	Yes	Yes	No

(1) Knecht *et al.* (2004)

(2) Benito-Peña *et al.* (2005)

(3) Gudin *et al.* (2001)

REFERENCES

ACFS 2006. Maximum Residues Limits Under Positive List System in Food Sanitation Law: Japan, 314-315.

Alfonta, L., Bardea, A., Khersonsky, O., Katz, E. and Willner, I. 2001. Chronopotentiometry and Faradaic impedance spectroscopy as signal transduction methods for the biocatalytic precipitation of an insoluble product on electrode supports: routes for enzyme sensors, immunosensors and DNA sensors. *Biosensors and Bioelectronics* **16**: 675-687.

Altria, K.D. 1999. Application of microemulsion electrokinetic chromatography to the analysis of a wide range of pharmaceuticals and excipients. *Journal of Chromatography A* **844**: 371-386.

Andersson, K., Hämäläinen, M. and Malmqvist, M. 1999. Identification an Optimization of Regeneration Conditions for Affinity-Based Biosensor Assays. A Multivariate Cocktail Approach. *Analytical Chemistry* **71**:2475-2481.

AOAC International 1996. Dairy products, Chapter 33, 38-43.

Bain, C.D. and Whitesides, G.M. 1988. Molecular-level control over surface order in self assembled monolayer films of thiols on gold. *Science* **24**: 62-63.

Bard, A.J. and Faulkner, L.R. 2001. Electrochemical methods; fundamentals and applications. New York, John Wiley & Sons, INC.

Bart, M., Stigter, E.C.A., Stapert, H.R., Jong, G.J. and Bennekom, W.P. 2005. On the response of a label-free interferon-γ immunosensor utilizing electrochemical impedance spectroscopy. *Biosensors and Bioelectronics* **21**: 49-59.

Baxter, G.A., Ferguson, J.P., O'Connor, M.C. and Elliot, C.T. 2001. Detection of Streptomycin residues in whole milk using an optical immunobiosensor. *Journal of Agricultural and Food Chemistry* **49**: 3204-3207.

Benito-Peña, E., Partal-Rodera, A.I., León-González, M.E. and Mereno-Bondi, M.C. 2006. Evaluation of mixed mode solid phase extraction cartridges for the preconcentration of beta-lactam antibiotics in waste water using liquid chlomatography with UV-DAD detection. *Analytical Chemica Acta* **556**: 415-422.

Berkow, R. and Talbott, J.H. 1977. The Merck Manual of Diagnosis and Therapy: New Jersey, 1822-1823.

Berggren, C. and Johansson, G. 1997. Capacitive measurements of antibody-antigen interactions in a flow system. *Analytical Chemistry* **69**: 3651-3657.

Bergstrom, C., Sternesjo, A., Bjurling, P. and Lofar, S. 1999. Design and use of general capturing surface in optical biosensor analysis of sulphamethazine in milk. *Food and Agricultural and Immunology* **11**: 329-338.

Berney, H., West, J., Haefele, E., Alderman, J., Lane, W. and Collins, J.K.2000. A DNA diagonostic biosensor: development, characterization and performance. *Sensors and Actuators B* **68**: 100-108.

Boatto, G., Cerri, R., Pau, A., Palomba, M., Pintore, G. and Denti, M.G. 1998. Monitoring of benzylpenicillin in ovine by HPLC. *Journal of Pharmaceutical and Biomedical Analysis* **17**: 733-738.

Bordi, F., Cametti, C. and Gliozzi, A. 2002. Impedance measurements of self-assembled lipid bilayer membranes on the tip of an electrode. *Bioeletrochem* **57**: 39-46.

Brillas, E., Cabot, P.L., Garrido, A., Montilla, M., Rodriguez, R.M. and Carrasco, J. 1997. Faradaic impedance behavior of oxidized and reduced poly(2,5-di-(2-thienyl)-thiophene) films. *Journal of Electroanalytical Chemistry* **430**: 133-140.

Brito, R.B. and Junqueira, R.G. 2006. Determination of Beta-Lactam Residues in Milk by High Performance Liquid Chromatography. *Brazillian Archives of Biology and Technology* **49**: 41-46.

Buck R.P. and Lindner, E. 1994. Recommendations for nomenclature of ion-selective electrodes (IUPAC Recommendations 1994). *Pure and Applied Chemistry*. **66**: 2527-2536.

Carter, R.M.,Jacobs, M.B., Lubrano, G.J. and Guibault, G.G. 1997. Rapid detection of alflatoxin B_1 with immunochemical optodes. *Analytical Letters* **30**: 1465-1482.

Castillo, J., Gáapár, S., Leth, S., Niculescu, M., Mortari, A., Bontidean, I., Soukharev, V., Domeanu, S.A., Ryabov, A.D. and Csöregi, E. 2004. Biosensors for life quality Design, development and applications. *Sensors and Actuators B* 102: 179-194.

Chan, P.H., Liu, H.B., Chen, Y.W., Chan, K.C., Tsang, C.W., Leung, Y.C. and Wong, K.Y. 2004. Rational Design of a Novel Fluorescent Biosensor for (-Lactam Antibiotics from a Class A (-Lactamase. Journal of the American Chemical Society **126**: 4074-4075.

Collings, A.F. and Caruso, F. 1997. Biosensors: recent advances. Repprts on Progress in Physics **60**: 1397-1445.

Dijiksma, M., Kamp, B., Hoogvliet, J.C. and Bennekom, W.P. 2001. Development of an Electrochemical Immunosensor for Direct Detection of Interferon-(at the Attomolar Level. Analytical Chemistry **73**: 901-907.

DeMarco, D.R., Saaski, E.W., McCrae, D.A. and Lim, D.V. 1999. Rapid detection of Escherichia coli O157:H7 in group beef using a fiber-optic biosensor. *Journal of Food Protection* **62**: 711-716.

Dubreuil, P., Daigneault, J., Couture, Y., Guay, P. and Landry, D. 2001. Penicillin concentrations in serum, milk, and urine following intramuscular and subcutaneous administration of increasing doses of procain penicillin G in lactating dairy cows. *The Canadian Journal of Veterinary Reasearch* **65**: 173-180.

EEC (1990) Council Regulation No 2377/90 of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. *Official Journal of the European Communities,* **L224**: 1-8.

Eggins, B.R. 1996. *Biosensors: an introduction.* Chichester: John Wiley & Sons Ltd.

Emaldi, P., Fapanni, S. and Baldini, A. 1995. Validation of capillary electrophoresis method for the determination of cephradine and its related impurities. *Journal of Chromatgraphy A* **711**: 339-346.

Eppelsheim, C., Aubeck, R. and Hampp, N. 1995. Comparision of potentiometric enzyme sensors for urea and penicillin-G: differential thick-sensors versus classical electrodes. *Journal of Membrane /science* **100**: 131-137.

Eurachem 1998. The fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics, update on December, 1998.

Frère, J.M. and Joris, B. 1985. Penicillin-sensitive enzymes in peptidoglycan biosynthesis. *CRC Critical Reviews in Microbiology* **11**: 299-396.

Furusawa, N. 2000. Rapid liquid chromatographic determination of residual penicillin G in milk. *Fresenius Journal of Analytical Chemistry* **368**: 624-626.

Gaudin, V., Fontaine, J. and Maris, P. 2001. screening of penicillin residues in milk by a surface plasmon resonance-based biosensor assay : comparison of chemical and enzymatic sample pre-treatment. *Analytical Chimica Acta* **436**: 191-198.

Gaudin, V. and Maris, P. 2001. Development of a iosensor-based immunoassay for screening of chloramphenicol residues in milk. *Food and Agricultural and Immunology* 13: 77-86.

Ghidini, S., Zanardi, E., Varisco, G. and Chizzolini, R. 2002. Prevalaence of Molecules of (-Lactam Antibiotics in Bovine Milk in Lombardia and Emilia Romagna (Italy). Ann. Fac. Medic. Vet. di Pharma **22**: 245-252.

Ghindilis, A.L., Atanasov, P., Wilkinst, M. and Wilkins, E. 1998. Immunosensors: electrochemical sensing and other engineering approaches. *Biosensors & Bioelectronics* **13**: 113-131.

Ghindilis, A.L., Skorobogat'ko, O.V. and Yaropolov, A.I. 1991. Immunopotentiometric electrodes based on bioelectrocatalysis in the absence of mediators. *Biomedical Sciences* **2**:520-525.

Greko, C. 1996. Antibiotika och resistens-ur diurhälsoperspektiv. *Skogs-och Lantbruksakademiens tidsskrift* **135**: 27-35.

Greko, C and Sternberg, S. 1996. *FAKTA Verterinärmedicin,* Swedish University of Agricultural Sciences,Vol. 11.

Grunwald, l. and Petz, M. 2003. Food processing effects on residues: penicillins in milk and yoghurt. Analytical Chimica Acta **483**: 73-79.

Guan, J.G., Miao, Y.Q. and Zhang, Q.J. 2004. Impedimetric Biosensors. *Journal of Bioscience and Bioengineering* **97**: 219-226.

Guiducci, C., Stagni, C., Zuccheri, G., Bogliolo, A., Benini, L., Samor, B. and Ricco, B. 2004. DNA detection by integrable electronics. *Biosensors and Bioelectronics* **19**: 781-787.

Gustavsson, E., Bjurling, P. and Sternesjö, Á. 2002. Biosensor analysis of penicillin G in milk based on the inhibition of carboxypeptidase activity. *Analytica Chimica Acta* **468**: 153-159.

Gustavsson, E. 2003. SPR Biosensor Analysis of β-Lactam Antibiotics in Milk. Doctoral Thesis of Agricultural Sciences, Swedish University, Uppsala, Sweden.

Gustavsson, E. 2004. SPR Biosensor Analysis of β-lactam Antibiotic in Milk. Doctoral Thesis of Agricultural Sciences, Swedish University, Uppsala, Sweden.

Gustavsson, E., Degelaen, J., Bjurling, P. and Sternesjo, A. 2004. Determination of β-lactam in milk Using a Surface Plasmon Resonance-Based Biosensor. Journal of Agricultural and Food Chemistry **52**: 2791-2796.

Haasnoot, W. and Verheijen, R. 2001. A direct (non-competitive) immunoassay for gentamicin residues with an optical biosensor. *Food and Agricultural and Immunology* **13**: 131-134.

Hamid, I.A., Ivnitski, D., Atanasov, P. and Wilkins, E. 1999. Highly sensitive flow-injection immunoassay system for rapid detection of bacteria *Analytical Chimica Acta* **399**: 99-108.

Hermanson, G.T., Mallia, A.K. and Smith, P.K. 1992. Immobilized affinity ligand techniques. Rrinted in the United States of Americas. Academic Press, Inc. 1250 Sixth Avenue, San Diego, California

Harris, R.D., Luff, B.J., Wilkinson, J.S., Pichier, J., Brecht, A., Gauglitz, G. and Abuknesho, R.A. 1999. Integrated optical surface plasmon resonance immunoprobe for simazine detection. *Biosensors and Bioelectronics* **14**: 377-386.

Hleli, S., Martelet, C., Abdelghani, A., Bessueille, F., Errachid, A., Samitier, J., Hays, H.C.W., Millner, P.A., Burais, N. and Jaffrezic-Renault, N. 2006. An immunosensor for haemoglobin based on impedimetric properties roperties of a new mixed self-assembled monolayer. *Materials Science and Engineering C* **26**: 322-327.

Janek, R.P., Fawcett, W.R. and Ulman, A.1997. Impedance spectroscopy of self-assembled monolayers on Au (III): evidence for complex double-layer structure in aqueous at the potential of zero charge. *Journal of Physical Chemistry* **101**: 4550-4558.

Jiang, D.C., Tang, J., Liu, P.Y., Shen, X.R. and Kong, J.L. 2003. Covalently coupling the antibody on an amine-self-assembled gold surface to probe hynluroman-binding protein with capacitance measurement. *Biosensors & Bioelectronics* **18**: 1183-1191.

Johansson, M.A. 2004. Immunosensor methods for drug residue control of food. Doctoral Thesis of Agricultural Sciences, Swedish University, Uppsala, Sweden.

Kaneene, J.B. and Ahl, A. 1987. Drug residues in dairy cattle industry: epidemiological evaluation of factors influencing their occurrence. *Journal of Dairy Science* **70**: 1691-1699.

Katz, E., Alfonta, L. and Willner, I. 2001. Chronopotentiometry and Faradaic impedance spectroscopy as methods for signal transduction in immunosensors. *Sensors and Actuators B* **76**: 134-141.

Katz, E. and Willner, I. 2003. Probing Biomolecular Interactions at Conductive and Semiconductive Surfaces by Impedance Spectroscopy: Routes to Impedimetric Immunosensors, DNA-Sensors, and Enzyme Biosensors. *Electroanalysis* **15**: 913-947.

Kharitonov, A.B., Alfonta, L., Katz, E. and Willner, I. 2000. Probing of bioaffinity interactions at interfaces using impedance spectroscopy and chronopotentiometry. *Journal of Electroanalytical Chemistry* **487**: 133-141.

Knecht, B.G., Strasser, A., Dietrich, R., Märtlbauer, E., Niessner, R. and Weller, M.G. 2004. Automated Microarray System for the Simutaneous Detection of Antibiotics in milk. *Analytical Chemistry* **76**: 646-654.

Lagane, B., Treilhou, M. and Couderc, F. 2000. Capillary electrophoresis: theory, teaching approach and separation of oligosaccharides using indirect UV detection. *Biochemistry and Molecular Biology Education* **28**: 251-255.

Lapierre, A., Olsina, R.A. and Raba, J. 1999. Continuous-flow/stopped-flow system incorporating a rotating bioreactor for the determination of penicillins in pharmaceutical products. *Analytica Chimica Acta* **396**: 143-149.

Leszczyńska, E., Glab, S., Soköl, A., Dziegielewaki, K., Rokicka, R. and Konchi, R. 1998. Potentiometric biosensor for control of biotechnological production of penicillin G. *Analytica Chimica Acta* **368**: 205-210.

Li, Y.M., Schepdael, A.V., Zhu, Y., Roets, E. and Hoogmartens, J. 1998. Development and validation of amoxicillin determination by micellar electrokinetic capillary chromatograohy. *Journal of Chromatography A* **812**: 227-236.

Lihl, S., Rehorek, A. and Peta, M. 1996. High-performance liquid chromatographic determination of penicillins by automated solid-phase extraction and photochemical degradation with electrochemical detection. *Journal of Chromatography A* **729**: 229-235.

Limbut, W. 2006. Affinity Biosensor Using Electrochemical Detection Principle. Doctoral Thesis of Philosophy in Chemistry Prince of Songkla University, Thailand.

Limbut, W., Kanatharana, P., Mattiasson, B., Asawatreratanakul, P. and Thavarungkul, P. 2006. A comparative study of capacitive immunosensors based on self-assembled monolayers formed thiourea, thioctic acid, and 3-mercaptopropionic acid. *Biosensors & Bioelectronics* **22**: 233-240.

Loksuwan, J. and Tomongkol, T. 2005. HPLC Determination of penicillin G and cloxacillin residues in milk and the stability of residues in milk after heating. *31st Congress on Science and Technology of Thailand at Suranaree University of Technology* 18-20 October.

Long, G and Winefordner, J.D. 1982. Limit of Detection. *Analytical Chemistry* **55**: 712A-724A.

Luong, J.H.T., Bouvrette, P. and Male, K.B. 1997. Developments and applications of biosensors in food analysis. *TIBTECH SEPTEMBER* **15**: 369-377.

Luppa, P.B., Sokoll, L. and Chan, D.W. 2001. Review Immunosensors-principles and applications to clinical chemistry. *Climica Chimica Acta* **314**: 1-26.

Marchetti, M., Schwaiger, I. and Schmid, E.R. 2001. Determination of benzylpenicillin, oxacillin, cloxacillin and dicloxacillin in cows' milk by ion-pair high-performance liquid chromatography after precolumn derivatization. *Fresenius Journal of Analytical Chemistry* **371**: 64-67.

Maupas, H., Soldatkin, A. P., Martelet, C., Jaffrezic-Renault, N. and Mandrand, N. 1997. Direct Immunosensing using Differential Electrochemical Measurement of Impedimetric Variations. *Journal of Electroanalytical Chemistry* **421**:165-171.

Medyantseva, E.P., Verlib, M.G., Budnikov, G.K. and Tyshlek, M.P. 1998. A new approach to selective detection of 2,4-dichlorophenoxyacetic acid with a cholinesterase amperometric biosensor. *Biochemistry and Microbiology* **34**: 202-205.

Mellgren, C. and Sternesjö, A. 1998. Optical immunobiosensor assay for determining enrofloxacin and ciprofloxacin in bovine milk. *Journal of AOAC International* **81**: 394-397.

Michalska, K., Pajchel, G. and Tyski, S. 2004. Capillary electrophoresis method for simultaneous determination of penicillin G, procaine and dihydrostreptomycin in veterinary drugs. *Journal of Chromatography B* **800**: 203-209.

Miller, E.L. 2002. The penicillins: A review and update. *Journal of Midwifery and Womens Health* **47**: 426-434.

Miller, J.C. and Miller, J.N., 1993. Statistics for Analytical Chemistry, third edition. Simon & Schuster International Group, West Sussex.

Msagati, T.A.M. and Nindi, M.M. 2007. Determination of β-lactam residues in foodstuffs of animal origin using supported liquid membrane extraction and liquid chromatography-mass spectrometry. *Food Chemistry* **100**: 836-844.

Nishi, H. and Terabe, S. 1995. Optical resolution of drugs by capillary electrophoretic techniques. *Journal of Chromatography A* **694**: 245-276.

Nozal, L., Arce, L., Ríos, A. and Valcárcel, M. 2004. Development of a screening method for analytical control of antibiotic residues by micellar electrokinetic capillary chromatography. *Analytica Chimica Acta* **523**: 21-28.

Nunes, G.S., Barceló, D., Grabaric, B.S., Diaz-Cruz, J.M. and Ribeiro, M.L. 1999. Evaluation of a highly sensitive amperometric biosensor with low cholinesterase change immobilized on a chemically modified carbon paste electrode for trace determination of carbamates in fruit, vegetable and water samples. *Analytica Chimica Acta* **399**: 37-49.

Orazio, P.D. 2003. Review Biosensors in clinical chemistry. *Clinica Chimica Acta* **334**: 41-69.

Ouerghi, O., Touhami, A., Jaffrezic-Renault, N., Martelet, C., Ouada, H.B. and Cosnier, S. 2002. Impedimetric immunosensor using avidin-biotin for antibody immobilization. *Bioelectrochemistry* **56**: 131-133.

Pajchel, G., Michalska, K. and Tyski, S. 2004. Application of capillary electrophoresis to the determination of various benzylpenicillin salts. *Journal of Chromatography A* **1032**: 265-272.

Palchetti, I., Cagnini, A., De Carlo, M., Coppi, C., Mascini, M. and Turner, A.P.F. 1997. Determination of anticholinesterase pepticides in real samples using a disposable biosensor. *Analytica Chimica Acta* **337**: 315-321.

Park, S-M. and Yoo, J-S. 2003. Electrochemical impedance spectroscopy for better electrochemical measurements. *Analytical Chemistry,* November 1:455A-461A.

Park, I.S., Kim, D.K., Adanyi, N., Varadi, M. and Kim, N. 2004. Development of a direct-binding chloramphenicol sensor based on thiol or sulfide mediated self-assembled antibody monolayers. *Biosensors and Bioelectronics* **19**: 667-674.

Park, J.T. and Strominger, J.L. 1957. Mode of action of penicillin. *Science* **125**: 99-101.

Pita, M.T.P., Reviejo, A.J., Villena, F.J.M. and Pingarron, J.M. 1997. Amperometric selective biosensing of dimethyl- and diethyl-dithiocarbamates based on inhibition process in a medium of reversed micelles. *Analytica Chimica Acta* **340**: 89-97.

Poghossian, A., Schönng, M.J., Schroth, P., Simonis, A. and Lüth, H. 2001. An ISFET-based penicillin sensor with high sensitivity, low detection limit and long lifetime. *Sensors and Actuators B* **76**: 519-526.

Poghossian, A., Thust, M., Schöning, M.J., Müller-Veggian, M., Kordos, P. and Lüth, H. 2000. Cross-sensitivity of a capacitive penicillin sensor combined with a diffusion barrier. *Sensors and Actuators B* **68**: 260-265.

Popelka, P., Nagy, J., Marcincak, S., Rozanska, H. and Sokol, J. 2004. Comparision of sensitivity of various screening assay and chromatography technique for penicillin residue detection in milk. Bulletin of the Veterinary Institute in Pulawy **48**: 273-276.

Puig, P., Borrull, F., Aguilar, C. and Calull, M. 2006. Sample stacking for the analysis of penicillins by microemulsion electrokinetic capillary chromatography. *Journal of Chromatography B* **831**: 196-204.

Rasooly, A. 2001. Surface plasmon resonance analysis of Staphylococcal enterotoxin B in food. *Journal of Food Protection* **64**: 37-43.

Rasooly, A. and Rasooly, L. 1999. Real time biosensor analysis of Stapphylococcal enterotoxin A in food. *International Journal of Food Microbiology* **49**: 119-127.

Ren, X. and Pickup, P.O. 1997. An impedance study of electron transport and electron transfer in composite polypyrrole + polystyrenesulphonate films. *Journal of Electroanalytical Chemistry* **420**: 251-257.

Rickert, J., Göpel, W., Beck, W., Jung, G. and Heiduschka, P. 1996. A "mixed" self-assembled monolayer for an impedimetric immunosensor. *Biosensors & Bioelectronics* **11**: 757-768.

Riediker, S., Diserens, J.M. and Stadler, R.H. 2001. Analysis of β-Lactam Antibiotics in Incurred Raw Milk by Rapid Test Methods and Liquid Chromatography Coupled with Electroapray Ionization Tandem Mass Spectrometry. *Journal of Agricultural and Food Chemistry* **49**: 4171-4176.

Riediker, S., Rytz, A. and Stadler, R.H. 2004. Cold-temperature stability of five β-lactam antibiotics in bovine milk and milk extracts prepared for liquid chromatography-electrospray ionization tandem mass spectrometry analysis. *Journal o Chromatography A* **29**: 359-363.

Sciacchitano, C.J., Mopper, B. and Specchio, J.J. 1994. Identification and separation of five cephalosporin by micellar electrokinetic capillary chromatography. *Journal of Chromatography B* **657**: 395-399.

Sharma, S.K., Sehgal, N. and Kumar, A. 2003. Biomolecules for development of biosensors and their applications. *Current Applied Physics* **3**: 307-316.

Skladal, P. and Kalab, T. 1995. A multichannel immunochemical sensor for determination of 2,4-dichlorophenoxyacetic acid. *Analytica Chimica Acta* **316**: 73-78.

Starodub, N.F., Dzantive, B.B., Starodub, V.M. and Zherdev, A.V. 2000. Immunosensor for the determination of the herbicide simazine based on an ion-selective-field-effect transistor. *Analytica Chimica Acta* **424**: 37-43.

Steegborn, C. and Skládal, P. 1997. Construction and characterization of the direct piezoelectric immunosensor for atrazine operating in solution. *Biosensors and Bioelectronics* **12**: 19-27.

Sternesjö, ´., Mellgren, C. and Björck, L. 1995. Determination of Sulfamethazine Residues in milk by a Surface Plasmon Resonance-Based Biosensor Assay. *Analytical Biochemistry* **226**: 175-181.

Stred'anský, M., Pizzariello, A., Stred'anská, S. and Miertus, S. 2000. Amperometric pH-sensing biosensors for urea, penicillin, and oxalacetate. *Analytica Cemica Acta* **415**: 151-157.

Swartz, M.E. and Krull, I.S. 1997. Analytical method development and validation. New York, Marcel Dekker, Inc.

Takeba, K., Fujinuma, K., Miyazaki, T. and Nakazawa, H. 1998. Simulaneous determination of β-lactam antibiotics in milk by ion-pair liquid chromatography. *Journal of Chromatography A* **812**: 205-211.

Tang, D., Yuan, R., Chai, Y., Dai, J., Zhong, X. and Liu, Y. 2004. A Novel Immobilization of Hepatitis B Surfane Antibody on Platinum Electrode Modified Colloidal Gold and Polyvinyl Butral as Matrices *via* Electrochemical Impedance Spectroscopy. *Bioelectrochemistry* **65**:15-22.

Tarbin, J.A., Farrington, W.H.H. and Shearer, G. 1995. Determination of penicillins in animal tissues at trace residue concentrations Part I. Determination of benzylpenicillin in milk by reversed-phase liquid chromatography with solid phase extraction and liquid chromatographic fractionation clean-up. *Analytica Chiimica Acta* **318**: 95-101.

Taverniers, I., Loose, M. D., and Bockstaele, E. V. 2004. Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. *Trends in Analytical Chemistry* **23**: 535-552.

Thévenot, D.R., Toth, K., Durst, R.A. and Wilson, G.S. 2001. Electrochemical biosensors: recommended definitions and classification. *Biosensors & Bioelectronics* **16**: 121-131.

Thévenot, D.R., Toth, K., Durst, R.A. and Wilso, G.S. 1999. Electrochemical biosensors: recommended definitions and classification. *Pure and Applied Chemistry* **77**: 2333-2348.

Traynor, I.M., Crooks, S.R.H.,Bowers, J. and Elliott, C.T. 2003. Detection of multi-(-agonist residues in liver matrix by use of a surface plasma resonance biosensor. *Analytica Chimica Acta* **483**: 187-191.

van der Merwe, P.A. 2000. Surface Plasmon Resonance. In Protein-Ligand interactions: A Practical Approach, 828 pp. Oxford University Press.

Vaughan, R.D., O'Sullivan, C.K. and Guibault, G.G. 1999. Sulfur based self-assembled monlayers (SAM's) on piezoelectric crystals for immunosensor development. *Fresenius Journal of Analytical Chemistry* **364**: 54-57.

Vikinge, T.P., Askendal, A., Liedberg, B., Lindahl,T. and Tengvall, P. 1998. Immobilized with surface plasmon resonance (SPR). *Biosensors and Bioelectronics* **13**: 1257-1262.

Wadu-Mesthrige, K., Amro, N.A. and Liu, G.Y. 2000. Immobilization of proteins on self-assembled monolayers. *Scanning* **22**: 380-388.

Wang, J. 2000. *Analytical Electrochemistry* (2nd ed). New York: John, Willey & Sons.

Wang, J., Carmon, K.S., Luck, L.A. and Sunl, I.I. 2005. Electrochemical Impedance Biosensor for Glucose Detection Utilizing a Periplasmic *E. coli* Receptor Protein. *Electrochemical and Solid-State Letters* 8: H61-H64.

Wang, J., Profitt, J.A., Pugla, M.J. and Sunl, I.I. 2006. Au Nanoparticle Conjugation for Impedance and Capacitance Signal Amplification in Biosensors. *Analytical Chemistry* **78**: 1769-1773.

Wang. M., Wang, L., Wang, G., Ji, X., Bai, Y., Li, T., Gong, S. and Li, X. 2004. Application of impedance spectroscopy for monitoring colloid Au-enhanced antibody immobilization and antibody-antigen reactions. *Biosensors and Bioelectronics* **19**; 575-582.

Weime,B. 1992. Can Antibiotic Test Kits Be Useful? PhD of Nutrition and Food Sciences, Utah State University, Logan, Utah.

Willner, I., Blonder, R. and Dagan, A. 1994. Application of photoisomerizable antigenic monolayer electrodes as reversible amperometric immunosensors. *Journal of the American Chemical Society* **116**: 9365-9366.

Wrobel, N. 2001. Optimization of Interfaces for Genosensors Based on Thiol Layers on Gold Films. Diploma Thesis of Chemie und Pharmazie der Universität Regensburg, Regensburg University, Regensburg.

Wu, Z.S., Li, J.S., Deng, T., Luo, M.H., Shen, G.L. and Yu, R.Q. 2005. A sensitive immunoassay based on electropolymerized films by capacitance measurements for direct detection of immunospecies. *Analytical Biochemistry* **337**: 308-315.

Yang, D.F., Wilde, C.P. and Morin, M. 1996. Electrochemical desorption and adsorption of nonyl-mercaptan at gold sigle crystal electrode surface. *Langmuir* **12**: 6570-6577.

Ye, J.M., Letcher, S.V. and Rand, A.G. 1997. Piezoelectric biosensor for detectionof Salmonella typhimurium. *Journal of Science* **62**: 1067-1071.

Yulaev, M.F., Sitdikov, R.A., Dmitrieva, N.M., Yazynina, E.V., Zherder, A.V. and Dzantiev, B.B. 2001. Development of a potentiometric immunosensor for herbicide simazine and its application for food testing. *Sensors and Actuators B* **75**: 129-135.

Zeng, S. S., Escobar, E. N. and Brown-Crowder, I. 1996. Evaluation of screening tests for detection of antibiotic residues in goat milk. *Small Ruminant Research* **21**: 155-160.

VITAE

Name Miss Supaporn Dawan

Student ID 4622115

Education Attainment

Degree	Name of Institute	Year of Graduation
Bachelor of Science in Education (Chemistry)	Prince of Songkla University	1999

Scholarship Awards during Enrolment

Higher Education Development Project: Postgraduate Education and Research Program in Chemistry (PERCH), funded by the Royal Thai Government.

List of Presentations

Oral Presentations

Supaporn Dawan, Pornpun Phimol, Proespichaya Kanatharana and Panote Thavarungkul. "Label-free Immunosensor based on Impedance Measurement". The 32nd Congress on Science and Technology of Thailand (STT32), Queen Sirikit National Convention Center, Bangkok, Thailand. 10th -12th October 2006

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