Comparative Study of Oxygen Electrode and Thermistor for Determination of Glucose

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Thesis Title	Comparative Study of Oxygen Electrode and Thermistor for Determination of Glucose
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ชื่อวิทยานิพนธ์ ศึกษาเปรียบเทียบการใช้ออกซิเจนอิเล็กโทรดและเทอร์มิสเตอร์สำหรับหาปริมาณกลูโคส

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บทคัดย่อ

งานวิจัยนี้ศึกษาเปรียบเทียบประสิทธิภาพของออกซิเจนอิเล็กโทรดและเทอร์มิสเตอร์ที่ใช้เป็นตัวตรวจวัดในระบบไบโอเซนเซอร์สำหรับใช้หาปริมาณกลูโคสในตัวอย่างพลาสมา โดยตรึงเอนไซม์กลูโคสออกซิเดสด้วยพันธะโควาเลนต์บนซิลิกาเจล ระบบแอมเพอโรเมทริกออกซิเจนอิเล็กโทรดใช้ในการวัดปริมาณของออกซิเจนที่เปลี่ยนแปลงไปจากปฏิกิริยาออกซิเดชันของกลูโคส ส่วนระบบแคลอริเมทริกใช้เทอร์มิสมิสเตอร์เป็นตัวตรวจวัดความร้อนที่เกิดขึ้น ในระบบหลังนี้ศึกษาโดยเปรียบเทียบเอนไซมีรีเอกเตอร์ที่มีการตรึงเอนไซม์กลูโคสออกซิเดสร่วมกับคะตะเลสกับเอนไซมีรีเอกเตอร์ที่มีการตรึงเอนไซม์เอนไซม์กลูโคสออกซิเดสเพียงอย่างเดียว

เปรียบเทียบประสิทธิภาพของตัวตรวจวัดทั้งสองด้วยระบบโฟลอินเจกชัน โดยใช้ไดอะไลเซอร์เพื่อป้องกันไม่ให้โมเลกุลขนาดใหญ่ผ่านเข้าไปอุดตันคอลัมน์รีเอกเตอร์ หาสภาวะที่เหมาะสมเพื่อให้ได้ค่าการตอบสนองสูงและเวลาในการวิเคราะห์น้อย ในระบบแอมเพอโรเมทริกขีดจำกัดการตรวจวัดอยู่ที่ความเข้มข้น 0.1 มิลลิโมลาร์ และมีการตอบสนองเชิงเส้นถึง 6.0 มิลลิโมลาร์ ส่วนเวลาที่ใช้ในการวิเคราะห์ 8-10 นาที สำหรับในระบบแคลอริเมทริกเอนไซมีรีเอกเตอร์ที่ใช้กลูโคสออกซิเดสตรึงร่วมกับคะตะเลสและใช้กลูโคสออกซิเดสเพียงชนิดเดียวมีขีดจำกัดการตรวจวัดและช่วงการตอบสนองเชิงเส้น 0.1-8.0 และ 0.3-8.0 มิลลิโมลาร์ ตามลำดับ ส่วนเวลาที่ใช้ในการวิเคราะห์มีค่าเท่ากันคือ 8-9 นาที เปรียบเทียบผลที่ได้จากการหาปริมาณกลูโคสในตัวอย่างพลาสมาด้วยเทคนิคทางไบโอเซนเซอร์กับวิธีมาตรฐานสองวิธี (Hexokinase method และ Glucose Sigma Kit) ทดสอบทางสถิติโดยใช้วิธี Regression line และ Wilcoxon signed rank test พบว่าผลการวิเคราะห์ด้วยวิธีการทางไบโอเซนเซอร์ทั้งสองระบบกับวิธีทาง Glucose Sigma kit ให้ผลที่สอดคล้องกัน แต่ทั้งสองวิธีนี้ให้ผลการทดสอบที่มีค่าสูงกว่าเทคนิคทาง Hexokinase method

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Abstract

This work compared the efficiency of the oxygen electrode and thermistor to determine glucose in plasma samples. Glucose oxidase was immobilized on silica gel by covalent binding. The glucose oxidase catalyzed the oxidation reaction of glucose into gluconolactone and hydrogen peroxide. The amperometric oxygen electrode was used to detect the change of oxygen in sample solution. In calorimetric system, themistor was used to detect the heat change. In the calorimetric system, enzyme reactor columns with different immobilized enzymes were studied. The column contained glucose oxidase co-immobilized with catalase was compared to one that only had immobilized glucose oxidase.

The evaluation of the two biosensors was done in the flow injection system that used a dialyser to filter off large molecules, thus, preventing them from blocking the enzyme reactor. They were optimized to obtain a high response in a short period of time. In the amperometric system, the limit of detection was 0.1 mM and the response was linear up to 6.0 mM. The analysis time was 8-10 min. In the calorimetric system, the linear range of the enzyme reactor column with glucose oxidase co-immobilized with catalase and immobilized glucose oxidase were 0.1-8.0 mM and 0.3-8.0 mM, respectively. The analysis time was 8-9 min. The biosensor systems were used to determine glucose in human plasma and validated the results by comparing to the hexokinase method and the commercial glucose Sigma kit. The results were statistically tested using the regression line and Wilcoxon signed rank tests.

Good agreement was obtained between the two biosensor techniques, amperometric and calorimetric, and between the commercial glucose Sigma kit and the biosensor systems. However, these biosensor techniques and the glucose Sigma kit, gave significantly higher results than the hexokinase method.

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Parichat Untang

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Chapter 1

Introduction

1.1 Introduction

A biosensor is a device which incorporates a biological sensing element such as enzyme, cell, tissue, antigen and antibody, with an appropriate transducer to give a sensing system specific to a target analyte (Cooper and Mcneil, 1990). Transducers are the physical component of the sensors that respond to the products of the biosensing process and give the response in a form that can be amplified, stored or displayed. Biosensor may be considered under a number of headings such as analyte or substrate (*i.e.,* glucose sensor, urea sensor, or lactate sensor), biological sensing element (*i.e.,* enzyme sensor, microbial sensor, or immunosensor) or transducers (*i.e.,* amperometric biosensor, potentiometric biosensor, or conductimetric biosensor).

In the biological sensing element section, enzyme is the most used because enzyme catalytic actitivity is highly selectivity for a given substrate (Kaisheva *et al.*, 1992; Cristina *et al.*, 2002). Within these enzyme-based biosensors several oxidase enzymes have been employed such as glucose oxidase, galactose oxidase, amino oxidase or ascorbate oxidase. These enzymes catalyze the oxidation reactions of analytes and the enzymatic reaction is shown in equation (1) (Kaisheva *et al.*, 1992; Peteu *et al.*, 1996).

$$\text{substrate} + O_2 \xrightarrow{\text{enzyme}} \text{product} + H_2O_2 \qquad (1)$$

The choice of a transducer uses in a biosensor depends on the reaction type and substances liberated or consumed (Tran Minh, 1993). The increase in hydrogen peroxide concentration or the decrease of oxygen concentration in reaction (1) can be detected electrochemically, both of them being proportional to the substrate concentration. To detect the oxygen consumption, oxygen electrode has been employed. This is the principle behind the first glucose biosensor (Cunningham, 1998) and this is by far the most studied and developed biosensor application.

Another interesting parameter to be monitor in a biosensor system is heat. Biochemical catalytic reactions involving enzyme, in common with most other reactions, generate heat. This heat can be measured and this is used as the basis of thermistor biosensors (Jesperson, 1990). Thus, either an oxygen electrode or a thermistor can be used to determine the analyte in several reactions. From this reason, it would be interesting to compare, with the same analyte, the efficiency of these two transducers in biosensor systems.

For this comparative study, glucose is a suitable target analyte because its reaction involve oxygen and heat changes and it is an important analyte in several applications. Development of glucose monitoring systems is still very important due to the significance of glucose determination in analytical laboratories, clinical laboratories, monitoring of glucose level in fermentation reactors , glucose estimation in the food industry, and pharmaceutical process. Measurement of glucose concentration in clinical application is one of the most frequently encountered analyses. A significant part of this analysis is connected with the prophylaxis, control and care of diabetes mellitus.

Most glucose biosensors use glucose oxidase (GOD) to catalyze the oxidation of glucose, producing gluconolactone, hydrogen peroxide (H_2O_2) and heat (Bruce and Vicent, 1997; Mosbach and Danielsson, 1981).

$$\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \text{Gluconolactone} + H_2O_2 + \Delta H(-80\ \text{kJ mol}^{-1}) \quad (2)$$

The oxygen consumption or the temperature change can be detected by using an oxygen electrode and a thermistor respectively. The objective of this work is to compare the efficiency of both transducers for the determination of glucose in human plasma samples. To study this, both systems were developed by using enzyme reactor column in the flow system.

1.2 Literature reviews

1.2.1 Glucose biosensor

Biosensor is a device that can detect or quantify analyte of interest. Sensing occurs when there is an interaction between the target analyte and the biological sensing element (e.g. enzyme, cell, antigen, antibody or tissue). Specific interaction between the target analyte and the biological sensing element produces a physico-chemical change which is detected by the transducer (Cooper and Mcneil, 1990; Eggins, 1996). The transducer can take many forms depending upon the parameters being measured. Electrochemical, optical, pH, conductivity or thermal changes are the most common. Some of the most intense efforts in biosensor research are in subjects as diverse as clinical monitoring, food quality determination and environmental monitoring (Atanasov and Wilkins, 1994). Glucose is the most extensively studied biosensor since it can be applied in several fields. In clinical analysis, measurement of glucose concentration is one of the most frequently encountered analyses to treat diabetic mellitus.

Diabetes mellitus is characterized by a relative or absolute lack of insulin due to the destruction of the pancreas. The principal function of insulin is to control the blood glucose level and maintain it within an approximate range in human blood. A normal concentration of glucose in human plasma is between 4.4-6.2 mM. The loss of control of the blood glucose level is the

earliest manifestation of the disease (Atanasov and Wilkins, 1994).

Common methods for the determination of glucose can be classified as chemical and enzymatic methods (Hanson, 1993). Most of the chemical methods are based on oxidation-reduction reaction to form a color product that can be measured photometrically. These chemical techniques are no longer used because of their non-specific, complicacy and some of the reagent are also toxic. Enzymatic methods are now mostly used because they are more specific when compared with chemical methods. These methods are based on the use of enzyme to increase the reaction specificity for glucose. However these enzymatic methods use free enzyme as a reagent and significantly increase the cost of analysis (Hanson, 1993). Therefore, immobilized enzyme are now applied more frequently as they can be used incorporating with detector that are sensitive for the product of biocatalytic process to form a biosensor and these has been reported in several work (Koudelka-Hep *et al.*, 1993; Pfeiffer *et al.*,1993; Narang *et al.*, 1994; Milardovic *et al.*, 1997; Rinken *et al.*, 1998; Ramanathan *et al.*, 2001).

Glucose biosensors are generally based on the enzyme glucose oxidase (GOD) (Kaisheva *et al.*, 1992; Xie *et al.*, 1994; Gamburzev *et al.*, 1997; Milardovic *et al.*, 1997; Dzyadevich *et al.*, 1998; Jing-Juan and Hong-Yuan, 2000) due to its good solubility in aqueous media, high stability and high specificity to glucose (Wilson and Turner, 1992). These biosensors differ essentially in the immobilization methods or in the types of the transducers (Haouz and Stieg, 2002).

Glucose oxidase catalyzes the oxidation of glucose to gluconolactone (or gluconic acid), hydrogen peroxide (H_2O_2) and heat (Mosbach and Danielsson, 1981; Bruce and Vincent, 1997; Cunningham, 1998)

$$\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \text{Gluconolactone} + H_2O_2 + \Delta H\ (-80\ \text{kJ mol}^{-1}) \quad (3)$$

Gluconic acid

The choice of transducer uses in a biosensor depends on the reaction type and the substances liberated or consumed (Tran-Minh, 1993). Several transducers have been applied to detect the changes in the oxidation reaction of glucose (reaction (3)). The principles of these transducers include potentiometric (Seki *et al.*, 1998; Tinkilic *et al.*, 2002; Nagy *et al.*, 1973), optical (Trettanak and Wolfbeis, 1989; Meadows and Shultz, 1993, Rosenzweig and Kopelman, 1996), amperometric (Lobel and Rishpon, 1981; Olsson *et al.*, 1986; Ward *et al.*, 2002) and calorimetric systems (Gadd *et al.*, 1977; Kiba *et al.*, 1984; Xie *et al.*, 1993; Ramanathan *et al.*, 2001).

Potentiometric biosensor is an electrochemical method based on the measurement of the difference in potential between a working electrode and a reference electrode. The determination of the electrode potential gives a direct indication of the concentration of the analyte (Tran-Minh, 1993). The various potentiometric transducers for detecting glucose concentration include the glass electrode for measurement of pH or specific electrodes sensitive to ions. The glass pH electrode has been used to detect the change in pH due to the production of gluconic acid. Conventional pH glass electrodes were used for the preparation of enzyme-pH electrode by either entrapping the enzyme within polyacrylamide gels around the glass electrode or as a liquid layer trapped within a cellophane membrane. The pH response was almost linear from 10^{-4}-10^{-3} mol l^{-1} with a pH change about 0.85 per decade (Nilson *et al.*, 1973). Nevertheless these biosensors present poor sensitivity, selectivity and linearity of calibration curve (Nilson *et al.*,1973; Cunningham, 1998).

The ion selective electrode investigating glucose activity via two consecutive reactions. Hydrogen peroxide produces in the oxidation reaction of glucose liberates iodine in the peroxide (POD) catalyzed second reaction (reaction (4)).

$$H_2O_2 + 2I^- + 2H^+ \xrightarrow{POD} 2\,H_2O + I_2 \qquad (4)$$

The increase in the iodine activity at the electrode surface can be monitored with an iodine selective electrode. Nagy *et al.* (1973) described a self-contained electrode for glucose based on an iodine membrane sensor. This enzyme electrode was fabricated by placing a thin layer containing a mixture of immobilized glucose oxidase and peroxidase over an iodine sensitive membrane electrode. The iodine selective electrode is one of the most selective and sensitive of all potentiometric detectors. However, this system required a pretreatment of the blood sample to remove interfering reducing agent, such as ascorbic acid, tyrosine and uric acid (Nagy *et al.*,1973).

An optical sensor has also been used to measure glucose concentration. This device correlates change in concentration, mass, or number of molecules to direct change in the characteristics of light. For this method one of the reactants or products of the enzymatic reaction has to be linked to colorimetric, fluorescent indicator molecules (Macrose *et al.*, 1999). Usually, an optical fiber is used for guiding the light signals from the source to the detector (Trettanak and Wolfbeis, 1989; Moreno *et al.*, 1990; Meadows and shultz, 1993). For example, Rosenzweig and Kopelman (1996) used 1-2 μm optical fiber tips with a fluorescent ruthenium complex immobilized with glucose oxidase in a photopolymerized acrylamide reaction layer. The metal complex was excited continuously by laser source, fluoresced and was quenched by oxygen in the sample. As oxygen was consumed in the glucose reaction the emission detected increases due to a reduce level of quenching.

The response time of the sensor was only 2 s. However, the disadvantage of the system is the loss of fluorescence signal due to the decrease in the number of dye molecules available for sensing. The lifetime of the sensors is less than 4-6 days (Rosenzweig and Kopelman, 1996).

Amperometric detection is another type of transducer principle used for glucose monitoring and is based on the measurement of current from the electrons transfer in the oxidation reaction. The increase in hydrogen peroxide concentration and the decrease in oxygen concentration in this reaction can be detected amperometically, both being proportional to the glucose concentration (Gamburzev *et al.*, 1996).

In the case of hydrogen peroxide detection, the increase in hydrogen peroxide increase the anodic current due to hydrogen peroxide oxidation at the electrode surface.

$$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^- \qquad (5)$$

In this type of glucose biosensor a platinum electrode is widely used as a catalytic electrode (Yao, 1983; Wang and Chen, 1994; Allietta and Thevenot, 1998). A major disadvantage of the use of hydrogen peroxide electrode is the high positive potential applied (+600mV) leads to the anodic co-oxidation of other reducing substances such as ascorbic acid, uric acid, dopamine and acetaminophen (Jung and Wilson, 1996). Various efforts have been made to eliminate these electrochemical interference. A cellulose acetate membrane was placed in front of the electrode to improve the selectivity of glucose analysis (Pfeiffe *et al.*, 1993). This membrane excludes uric acid, ascorbic acid and most other potentially interfering substance while still allowing small molecules such as hydrogen peroxide to pass through. However, the cellulose acetate membrane is not truly selective for hydrogen peroxide but only decrease the permeation of large molecules and the membrane is very

expensive (Frieder *et al.*, 1987). Lobel and Rishpon (1981) eliminated a part of the interferences by using a negatively charged dialysis membrane, which reject up to 0.0852 mmol l^{-1} of ascorbic acid and 0.464 mmol l^{-1} of uric acid. However, high concentration of these substances and also glutathione and bilirubin still interfere with the glucose signal. To date, several hydrogen peroxide electrode glucose biosensor is still being developed although hydrogen peroxide anodic oxidation is not always diffusion controlled and its rate may limit the signal from the sensor (Velho *et al.*, 1987).

1.2.2 Oxygen electrode

The use of oxygen detecting electrode as a transducer for glucose monitoring was the first electrochemical method developed. The decrease in oxygen concentration due to the oxidation reaction of glucose is detected by an oxygen electrode. Oxygen is measured as a result of the electrochemical reduction of oxygen on a catalytic electrode according to reaction (6) (Xie *et al.*, 1994).

$$O_2 + 2\,H_2O + 4e^- \longrightarrow 4OH^- \qquad (6)$$

An oxygen electrode membrane biosensor consists of two parts, an oxygen selective electrode and immobilized glucose oxidase. These two parts are separated by a hydrophobic oxygen permeable membrane (Xie *et al.*, 1994; Atanasov and Wilkins, 1994). The most common oxygen sensor was first proposed by Clark and Lyons in 1962. A voltage of –0.7 V is applied between the platinum cathode and the silver anode, sufficient to reduce the oxygen, and the cell current which is proportional to oxygen concentration is measured. The concentration of glucose is then proportional to the decrease in current (oxygen concentration). The oxygen electrode has an oxygen permeable membrane (such as PTFE, polythene or cellophane) covering the electrodes. A layer of

enzyme glucose oxidase is placed over this and held in place with a second membrane, cellulose acetate membrane. The glucose solution and oxygen can penetrate the first membrane to react with the enzyme. Only the remaining oxygen can penetrate the second membrane to be measured at the electrode (Eggins, 1996). The Clark type oxygen electrodes are almost insensitive to all types of interfering substances, but they are obviously very sensitive to variation in the oxygen within the solution in contact with electrodes (Velho *et al.*, 1987). From this advantage it is evidenced that oxygen electrode is an interesting transducer to be used to determine of glucose.

1.2.3 Thermistor

Many enzyme catalyzed reactions are generating heat in the range of 25 to 100 kJ mol^{-1}, which may be used as a basis for measuring the analyte concentration (Mosbach and Danielsson, 1981). This represents the most generally applicable type of biosensor. The heat can be measured by a thermistor and with the selectivity of enzyme can be used as the basis for calorimetric biosensors. The thermal enzyme probe (TEP) was one of the first instrument developed. The original TEP device had the biological sensing element immobilized directly on the glass encasement of the thermistor (Danielsson and Mosbach, 1987). A protective membrane was added over the immobilized component. Unfortunately, the major part of the heat evolved in the enzymatic reaction was lost to the surrounding solution without being detected and the probe of this type exhibits long response times which result in low sensitivity (Batailard *et al.*, 1993).

A more effective approach in thermal sensing was demonstrated using the thermistor in conjunction with an enzyme reactor column incorporated into a flow system. In this configuration the reactor column and thermistor combination is commonly refered to as an enzyme thermistor or ET (Danielsson and Mosbach, 1987). A measuring thermistor was inserted in the

thermal insulator device, usually at the outlet of the enzyme column (Figure 1). Temperature measurement in this device was more efficient than with the TEP, thus improving sensitivity (Cunningham,1998).

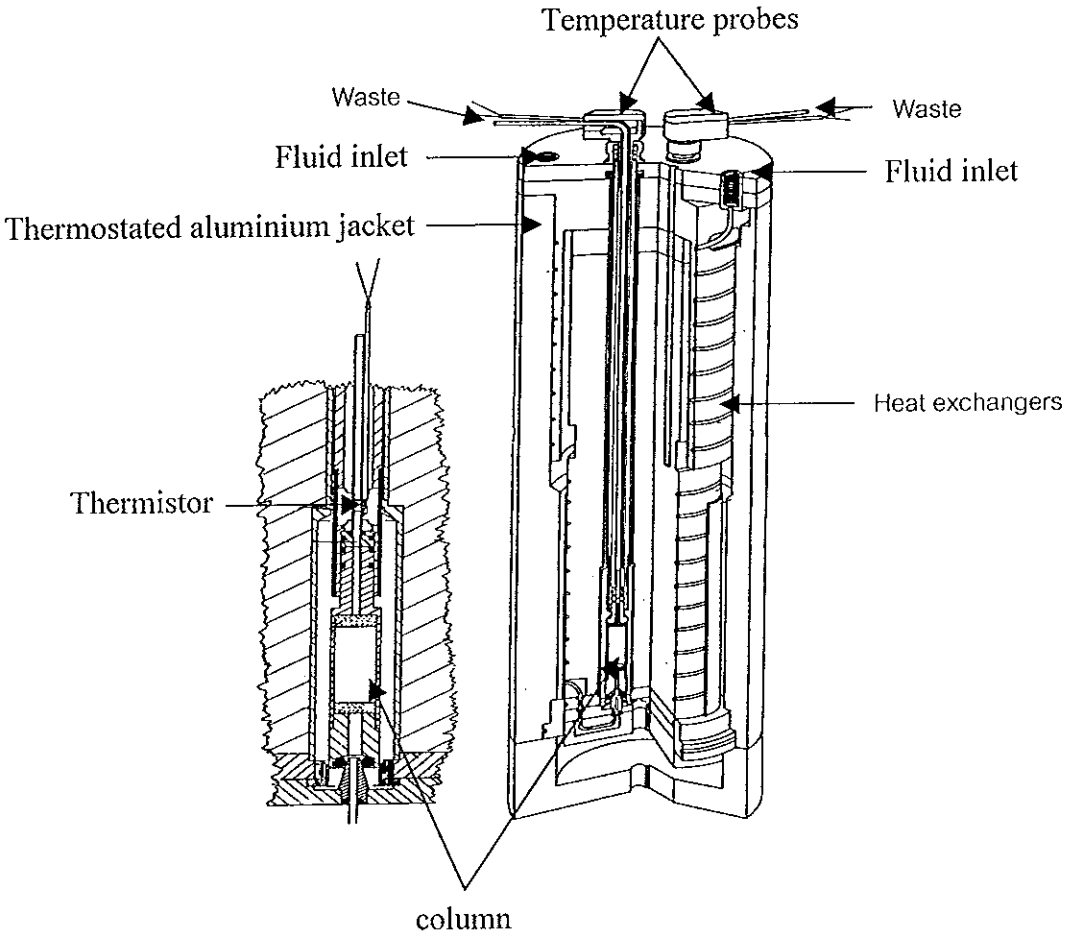


Figure 1 Cross-section of the calorimeter of an enzyme thermistor with an aluminium constant temperature jacket. The two identical column ports (one temperature probe with a column is shown) can be used independently or one of the ports can be used for a reference channel. The column attachment is illustrate on the left.
(From Danielsson, 1995)

Enzyme thermistor was developed to determine the analyte concentration in many laboratories (Rich *et al.*, 1979; Satoh *et al.*, 1981; Tse and Gough, 1987; Xie *et al.*, 1993; Ramanathan *et al.*, 2001). The device combined the selectivity of thermal biosensor with the flexibility of a flow system. For determining glucose concentration, either hexokinase or glucose oxidase has been used. The hexokinase was used in an injection analysis for glucose sample in a range of 0.5 to 50 mmol/l (McGlothlin and Jordan, 1975). The use of glucose oxidase instead of hexokinase was perferred due to better enzyme stability and no cofactor requirements (Danielsson and Mosbach, 1981). A disavantage was when using glucose oxidase the linearity was only up to 0.45 mmol/l glucose. This limitation can be overcome by diluting sample 10-100 fold or by injecting small serum volumes directly into the buffer entering the enzyme thermistor (Danielsson and Mosbach, 1987). Glucose concentrations determined by the enzyme thermistor agreed well with the values obtained from a conventional, spectrophotometric or enzymatic technique used in routine hospital diagnosis (Xie *et al.*, 1993; Harborn *et al.*, 1994; Harborn *et a.*, 1997). These enzyme thermistors have several advantages which are, no interference from by-product, no electrochemical or optical interferences, high operation stability of enzyme column and automated working with flow analysis (Ramanathan, 1999).

For an enzyme reactor column, enzyme is most often immobilized onto silanized glass beads using a crosslinked polymer matrix (e.g. glutaraldehyde) (Carlsson *et al.*, 1996; Harborn *et al.*, 1997). Recently Limbut (2001) showed that enzyme immobilized on silica gel by covalent binding had the same efficiency as the enzyme immobilized on CPG. Since silica gel is much cheaper than CPG it would be more cost effective to use silica gel inplace of CPG.

1.2.4 Flow system

Biosensors have undergo rapid development over the last few years. This is due to the combination of biological sensing element with the ever-growing number of transducers. The coupling of sensor with flow system is already a very popular option. The advantages normally cited are improved precision and accuracy, improved sensor lifetime and the entire analysis can be easily automated.

This approach was first reported used in an enzyme electrode system, in which a membrane containing immobilized enzyme is placed in front of the active surface of a electrode (Calvo and Danilowicz, 1997). However, the stability of an enzyme electrode is difficult to define because the use of quite a small amount of enzyme can result in the considerably lose of activity. This would cause a shift of the calibration curve downward (Guilbault, 1984). The reproducibility of such electrode is not very satisfactory and the interference to the determination of glucose usually can not be completely excluded, which greatly restricted its further development in practice (Xu and Chen, 2000).

To overcome this problem the enzymatic step and the detection step should be separated. This is readily feasible in a flow system, where the enzyme can be immobilized on a suitable support and incorporated into a column reactor, while quantification of product formed subsequently can be measured by the appropriate transducer (Hansen, 1994). With a reactor containing excess enzyme slight variations in the flow rate, pH, ionic strength or temperature and the presence of small concentration of inhibitors and activators will not affect the efficiency of the reactor (Marko-Varga, 1995). This is never the case with an enzyme electrode, where the amount of immobilized enzyme in membrane or on the electrode surface is more restricted. It will almost always be much too low to achieve equilibrium of the catalysed reaction within a reasonble time, particularly when used in flowing solution.

From the literatures cited, both oxygen electrode and thermistor are interesting transducers to be used for the determination of glucose. However, no one has compared these two transducers in the analysis of the same analyte. Therefore, it would be interesting to compare the efficiency of these two transducers in the biosensor system. In this study the comparison was done by observing several analytical parameters, such as sensitivity, linear range, limit of detection and dynamic characteristic of the response. Each of these systems used an enzyme reactor column in a flow analysis system to determine glucose in human plasma samples.

1.3 Objectives of the research

1. To develop and evaluate the performance of the amperometric and calorimetric glucose biosensor systems.
2. To use the biosensor systems to analyze glucose concentration in human plasma and compare with some standard methods.

1.4 Benefits

It is expected that these comparative study would provide useful information about the suitability of oxygen electrode or thermistor for the determination of glucose in a biosensor system.

1.5 Outline of the research

1. Immobilize enzyme on silica gel by covalent binding.
2. Optimize the operating condition in the flow-through biosensor system of both the amperometric and calorimetric systems.
3. Optimize the operating condition of biosensor in a flow-injection analysis system and compared this with the flow-through system.
4. Test the biosensor systems by determining glucose in human

plasma samples and compare the results with those obtained by using spectrophotometric system.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Biosensor technique

- D(+) – Glucose anhydrous ($C_6H_{12}O_6$, AR Grade: Fluka, Switzerland.)
- Glucose oxidase (EC 1.1.3.4 Type V-S : from *Aspergillus niger*, 1,300 units/ml: Sigma, USA)
- Catalase (EC 1.11.1.6: from Bovine Liver, 2,200 units/mg solid: Sigma, USA.)
- Silica gel (mean diameter 40-63 μm, mean pore diameter 60 Å, Merck, Germany.)
- 3-aminopropyl-triethoxysilane ($C_9H_{23}NO_3Si$, Biological Grade: Fluka, Switzerland.)
- Glutaraldehyde 25 % ($C_5H_8O_2$, Biological Grade: Electron Microscropy Science, USA.)
- Ethanolamine (C_4H_7NO, AR Grade: Merck, Germany.)
- Sodium azide (NaN_3 , AR Grade: Merck, Germany.)
- Sodium cyanoborohydride (CH_3BNNa, AR Grade: Fluka, Switzerland.)
- Sodium dihydrogenphosphate dihydrate ($NaH_2PO_4.2H_2O$, AR Grade: Merck, Germany.)
- Disodium hydrogenphosphate dihydrate ($Na_2HPO_4.2H_2O$, AR Grade: Merck, Germany.)
- Sodium hydroxide (NaOH, AR Grade: Merck, Germany.

- Sodium chloride (NaCl, AR Grade: BDH, England.)
- Hydrochloric acid 36.5-38 % (HCl, AR Grade: BHD, England.)
- Potassium dihydrogen orthophosphate (KH_2PO_4, AR Grade: BDH, England.)
- Tris-(hydroxymethyl)-aminomethan ($C_4H_{11}NO_3$, AR Grade: Fluka, Switzerland)

2.1.2 Glucose Sigma kit

- PGO Enzymes, 510-6
- Glucose Standard Solution, 635-100
- O-Dianisidine Dihydrochloride, 510-50

2.2 Apparatus

2.2.1 Biosensor technique

- Peristaltic pump (Minipuls 3, Gilson, France.)
- Syringe loading sample injection (Model 7125, Rheodyne, USA.)
- Microsyringe 500 µl (Code No.MS*R 500, Ito Corporation Fuji, Japan.)
- Dialyser (lab built, Biophysics Reseach Unit: Biosensor and biocurrent, Department of Physics, Faculty of Science, Prince of Songkla University.)
- Spectra pore 1 cellulose ester membrane, MWCO 6,000 (Spectrum Laboratories, USA.)
- YSI Model 5300 Biological Oxygen Monitoring (Yellow Spring Instrument Company, USA.)
- YSI Model 5331 Oxygen electrode (Yellow Spring Instrument Company, USA.)

- Enzyme thermistor (Department of Pure and Applied Biochemistry, Lund University, Lund, Sweden.)
- Chart recorder (Single channel Model 155, Linear Instrument Company, USA.)
- Microliter pipeter 200, 1000 and 5000 µl (Gilson, France.)

2.2.2 Glucose Sigma Kit

- Spectrophotometer (Model 6300, Jenway Ltd., U.K.)

2.3 Glucose biosensor

A biosensor is a device which incorporates a biological sensing element with an appropriate transducer to give a sensing system specific for the target analyte (Figure 1). The potential advantages of biosensors are numerous, most commonly cited are their low cost, fast response time, specificity of response, small size, convenience and the lack of need for sample preparation (Cooper and Mcneil, 1990).

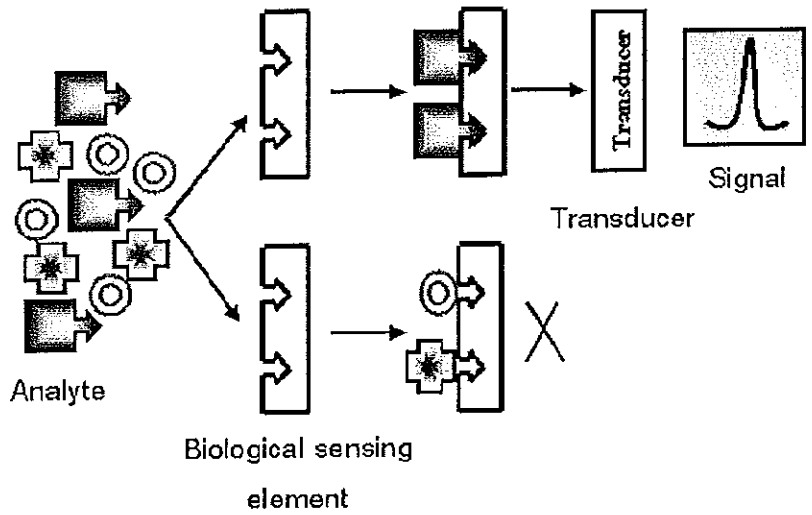


Figure 2 The biosensor: the biorecognition of biological material produces a signal that is detected by a transducer.

To determine glucose the enzyme glucose oxidase (GOD) is used as the biological material which catalyzes the oxidation of glucose to gluconolactone, hydrogen peroxide (H_2O_2) and heat (reaction 7)

$$\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \text{Gluconolactone} + H_2O_2 + \Delta H\ (-80\ \text{kJ mol}^{-1}) \qquad (7)$$

This enzyme-substrate reaction causes a change in the amount of oxygen and the temperature of the solution. An amperometric oxygen electrode can be used to detect the oxygen consumption while a thermistor can be used to detect the heat change. In a thermistor system the sensitivity can be improved by using the enzyme catalase (CAT) to further catalyze hydrogen peroxide (H_2O_2) into water, oxygen and heat (reaction 8) (Mosbach and Danielsson, 1981).

$$H_2O_2 \xrightarrow{\text{CAT}} \tfrac{1}{2} O_2 + H_2O + \Delta H\ (-100\ \text{kJ mol}^{-1}) \qquad (8)$$

From reactions (7) and (8) each mole of glucose would produce 180 kJ of total heat, thus the sensitivity of the glucose analysis using glucose oxidase can be increase by the co-immobilization of glucose oxidase and catalase

In this work we compare the efficiency of two transducers, that is, an oxygen electrode and an enzyme thermistor, to determine the concentration of glucose.

2.4 Immobilization of enzyme

Immobilized enzymes are defined as enzymes physically confined or localized in a certain region with retention of their catalytic activities and which can be used repeatedly and continuously (Chibata, 1978). Methods for enzyme immobilization can be classified into carrier-binding, cross-linking and

entrapping, as shown in Figure 3.

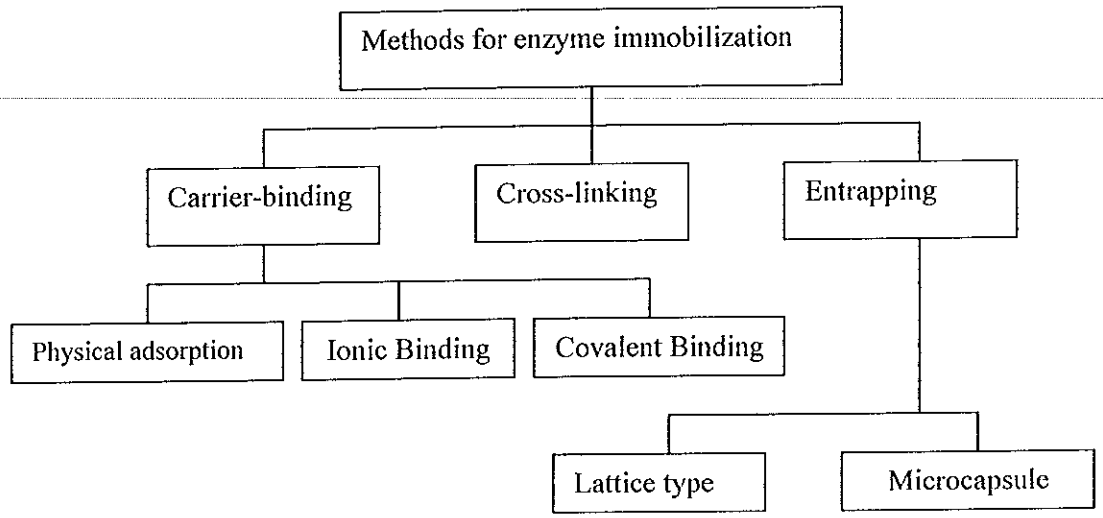


Figure 3 Methods for enzyme immobilization

Carrier–binding is subdivided into physical adsorption, ionic binding and covalent binding, while entrapping is divided into lattice type and microcapsule type. The disadvantages of physical adsorption and ionic binding methods are the binding forces between enzyme and carriers are less strong and leakage of enzyme from the carrier may occur in substrate solution of high strength or upon variation of pH compared with the covalent binding method.

In this work covalent binding between enzyme and silica gel was chosen. The immobilization of enzyme (glucose oxidase for amperomeric biosensor and glucose oxidase and catalase for calorimetric biosensor) on silica gel consists of a three-step procedure.

The initial step is to prepare the support materials, silica gel, by placing them in the oven at 110 °C for 3 hours. This is to get rid of any organic adsorbed to the support. It was then heated in 5% (w/v) nitric acid solution at 75°C for 45 minutes to remove any metal residues. The silica gel was rinsed thoroughly with distilled water and dried at 90°C overnight. The next step is the derivatization of the silica gel surface with organosilane. This was done by

adding 1 g of clean silica gel material into 18 ml of distilled water and 2 ml of 10% (v/v) 3–aminopropy–triethoxysilane. The pH of the mixture is adjusted to be between 3 and 4 with 6 M HCl. It was then heated at 75°C in a water bath for 2 hours. The silica gel was filtered on a Buchner funnel, and washed with 20 ml of distilled water and dried at 90°C overnight. The silica gel support may be stored at this point for later use. This procedure provides an alkylamine derivative on the surface of silica gel.

The final step is the coupling of protein (enzyme) to the alkylamine silica gel. The alkylamine silica gel was first activated by glutaraldehyde to yield aldehyde on alkylamine silica gel. This was done by adding 1.2 g (2 ml) alkylamine silica gel into 25 ml of 2.5% (v/v) glutaraldehyde in 0.05 M sodium phosphate buffer pH 7.00. The mixture was tumbled end over end for 60–90 minutes, during this time, the color of the carrier changed to orange–red. It was washed with 500 ml of distilled water and then with buffer repeatedly on Buchner funnel, until it has no odor of glutaraldehyde. It is important to remove all excess glutaraldehyde before adding enzyme, otherwise crosslinking will occur. The crosslinked enzyme will decrease the overall activity by blocking pores and preventing passage of larger molecules.

To immobilize the enzyme, 1.00 ml (1272 unit) of glucose oxidase was used in the amperometric biosensor and in the calorimetric biosensor 0.92 ml (1200 unit) of glucose oxidase and 71 mg (156,000 unit) of catalase were used. In both systems, enzymes was dissolved in 5 ml of 0.05 M sodium phosphate buffer pH 7.00 and added to 1 ml (sedimented volume) of activated alkylamine silica gel. The mixture was tumbled at room temperature (around 23°C). After 4–5 hours, 50 mg of sodium cyanoborohydride was added to reduce the Schiff's bond between aldehyde and enzyme, thus stabilizing the coupling. The mixture was tumbled again for another 15 hours and was then washed with 500 ml of buffer. After this 25 ml of 0.10 M ethanolamine pH 8.00 was added and the reaction was allowed for another 2 hours. This step was

to occupy all the aldehyde group which did not couple to the enzyme. The preparation was then washed with 500 ml of buffer and was packed into a small column reactor (7 mm inner diameter, 20 mm long for calorimetric system and 4 mm inner diameter, 30 cm long for amperometric system) to be used in the analytical process. When not used, the column reactor was stored in 0.05 M sodium phosphate buffer pH 7.00 + 0.02% sodium azide at 4°C.

2.5 Amperometric biosensor system

Amperometric sensor is a small electrochemical cell consists of two or three electrodes which are usually combined in a single body. A constant potential is applied to obtain a current response that is related to the concentration of the target analyte. This is accomplished by monitoring the transfer of electron(s) during the redox process of the analyte (Wang, 2000). This is the principle used in an oxygen electrode.

An oxygen electrode measures the concentration of dissolved oxygen in solution. The electrode consisted of 0.63 cm diameter Pt-cathode and Ag-anode encased in an epoxy block. Both electrodes are contained in a single body. The entire tip of electrode was covered with a gas-permeable membrane which contained a saturated KCl solution. Oxygen can be reduced at a cathode according to the following reaction.

$$O_2 + 2H_2O + 4e^- \rightleftharpoons 4OH^-$$

The saturated KCl allows the current to flow and silver chloride is formed on the silver anode.

$$Ag^o\,(metal) + Cl^- \rightleftharpoons AgCl + e^-$$

The current depends upon the rate of diffusion of oxygen to the cathode, and this rate is proportional to dissolved oxygen concentration. The current is

determined using an oxygen monitoring which was recorded by a chart recorder.

2.6 Calorimetric biosensor system

Biochemical reaction involving enzyme, in common with most other reactions, generate heat. This heat can be measured and this is used as the basis for thermistor biosensors. The thermistor, used to detect the temperature change, function by changing their electrical resistance with the temperature by equation (9)(Jespersen, 1990).

$$\ln\left[\frac{R_a}{R_b}\right] = K\left[\frac{1}{T_1} - \frac{1}{T_2}\right] \qquad (9)$$

Where R_a is the resistances of the thermistors at absolute temperature T_a.

R_b is the resistances of the thermistors at absolute temperature T_b.

K is characteristic temperature constant of the thermistor.

Since electrical resistance is the property which varies with temperature, thermistor are commonly incorporated into Wheatstone bridge circuits (Figure 4). Precise measurements of resistance may be obtained if the Wheatstone bridge is balanced manually so that no electrical potential exists between points A and B. In this balanced condition, it is known that

$$\frac{R_1}{R_T} = \frac{R_2}{R_3} \qquad (10)$$

Thus $V_1 = V_2$ (11)

and $V_1 = \frac{E_b R_1}{R_1 + R_2}$ (12)

$V_2 = \frac{E_b R_T}{R_3 + R_T}$ (13)

Where R_T is the resistance of a measuring thermistor

R_1 is the resistance of a reference thermistor

V_1 is the potential at A

V_2 is the potential at B

E_b is input bridge potential

R_2 and R_3 are resistances of adjustable resistans to give a balance bridge

In actual practice, it is very inconvenient, if not impossible, to balance the Wheatstone bridge, and therefore the unbalance potential is monitored (E_{un}). The expression relating the unbalance potential to the thermistor resistance is

$E_{un} = V_1 - V_2$ (14)

$E_{un} = E_b \left[\frac{R_1}{R_1 + R_2} - \frac{R_T}{R_3 + R_T} \right]$ (15)

where E_{un} is the unbalance potential

Therefore, the temperature change in the thermistor can be measured in the unbalance potential (E_{un}) form which is directly proportional to target analyte.

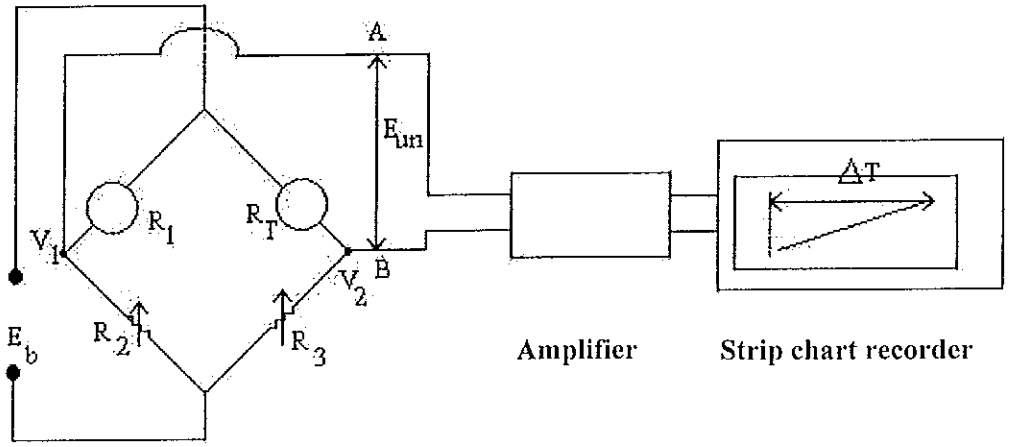


Figure 4 A Wheatstone bridge apparatus.

E_b is input bridge potential

E_{un} is unbalance potential

V_1 is the potential at A

V_2 is the potential at B

R_T is the resistance of the thermistor

R_1 is the resistance of a reference thermistor

R_2 and R_3 are resistances of adjustable resistans to give a balance bridge .

(from Jespersen, 1990)

An enzyme thermistor is shown in Figure 5. It consists of a temperature controlled aluminium cylinder set to either 30°C or 37°C in a polyurethan foam insulating cover. Another aluminium cylinder inside is linked up with the heat exchanger system to provide very stable temperature control. The enzyme column (Figure 6) is inserted into this thermostatted cylinder where a thermistor is attached to its outlet (Figure 5). There is also a reference thermistor in the same cylinder. The resistance is measured by a Wheatstone bridge and the unbalanced potential (E_{UN}) is recorded. The highest sensitivity of thermistor can be obtained at 0.001°C for an output signal of 100 mV.

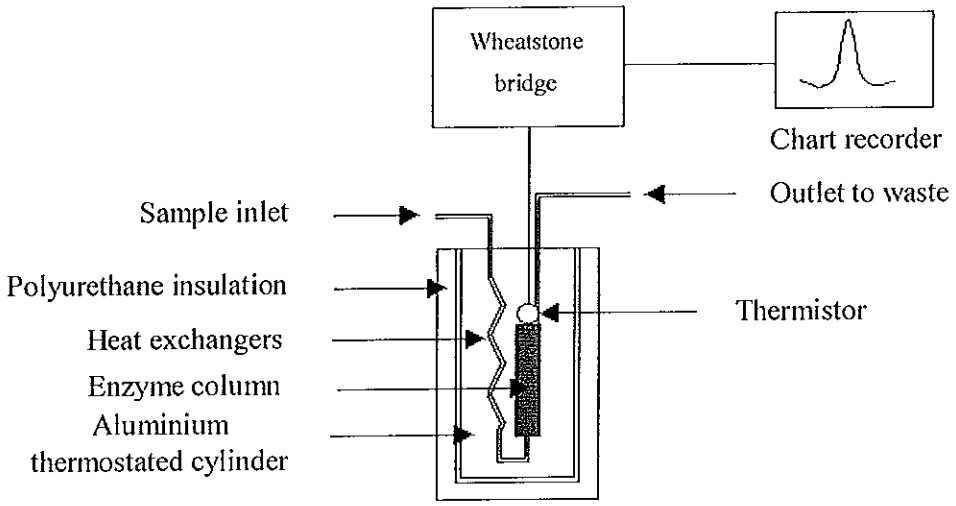


Figure 5 Schematic diagram of the calorimetric detection unit.

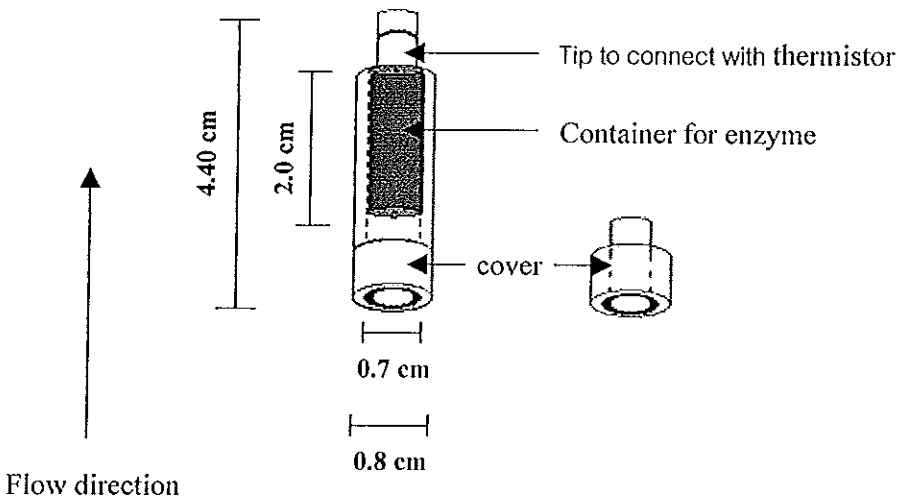


Figure 6 Schematic diagram of the enzyme column with a volume of 0.77 cm^3.

2.7 Instrumentation

In this work we applied the flow-through and flow-injection (FI) systems, where two types of transducer, amperometric and calorimetric, were used.The main objective of this work is to compare the efficiency of two transducers, that is, oxygen electrode and thermistor, to determine glucose concentration in plasma samples.

The flow-through amperometric and calorimetric systems are shown in Figures 7 and 8 respectively. Each system consists of

a) A propelling unit, a peristaltic pump, where steady flow rates of the solutions are controlled.

b) A sample loading unit where a constant volume sample solution is introduced into the analysis system. In the flow-through system, sample solutions were introduced as pulses in the continuous flow of buffer by switching the tube between buffer and sample containers.

c) A separation unit, a dialyser (Figure 9), this unit allows small molecules to pass through the membrane (cellulose ester MWCO 6000) and to be collected in the buffer on the other side of the membrane. Two dialysers with different diffusion areas, 1.5x298.0 mm^2 and 1.5x625.0 mm^2 were used in the amperometric and calorimetric systems respectively.

d) A reaction unit, a unit that the reaction will occur. In this work we used an enzyme reactor with immobilized glucose oxidase in the amperometric system and immobilized glucose oxidase and catalase in the calorimetric system.

e) A detection unit: In the amperometric system (Figure 10) this consisted of an oxygen electrode inserted into a chamber in the middle of a water insulator jacket. The contact volume between the electrode and the solution from the reaction unit was 115 μl. The

electrode monitored the change in oxygen of the solution due to the oxidation reaction of glucose. The water insulator jacket was used to control the temperature of the oxygen electrode.

For the calorimetric system this consisted of an enzyme thermistor (Figure 5) and a Wheatstone bridge. When the solution containing glucose passed through the enzyme column (Figure 6), heat was generated by the catalytic reactions of glucose and hydrogen peroxide. An increase in the temperature of the solution changed the resistance of the thermistor. This was detected by a Wheatstone bridge circuit and the unbalanced potential was recorded. Therefore, the change in temperature in this system was recorded as a voltage change.

In the flow-injection amperometric and calorimetric systems the only different is the sample loading unit. In this case a specific volume of sample was injected into an injection valve, placed in the sample carrier buffer line before the dialyser, before introduced into the sample carrier buffer.

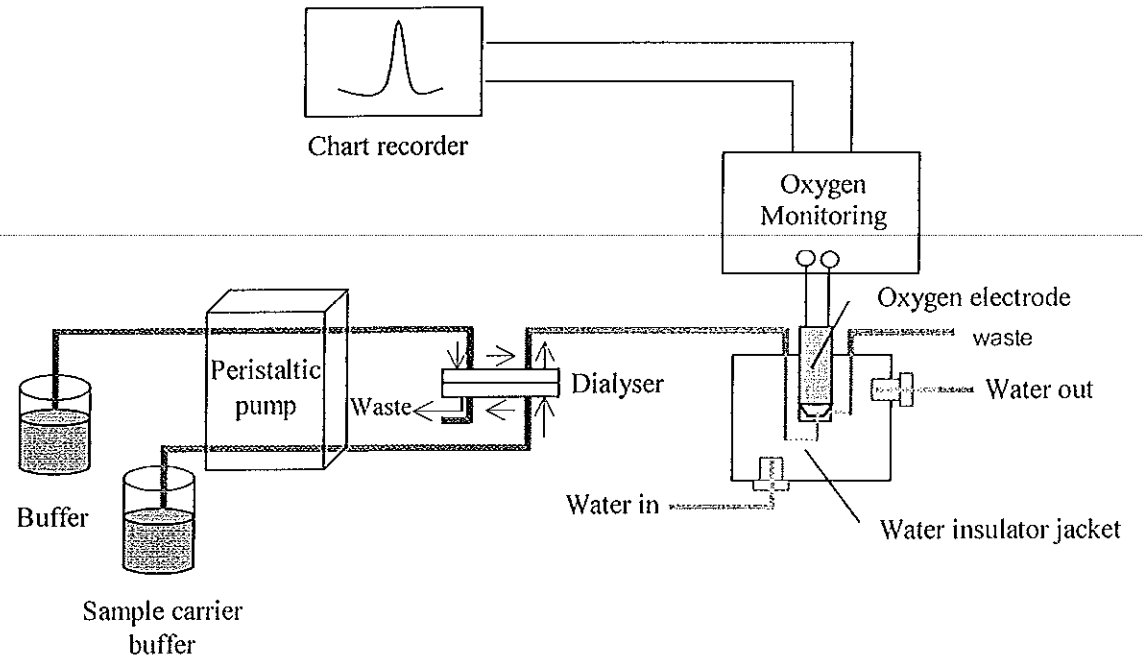


Figure 7 Schematic diagram of the amperometric system.

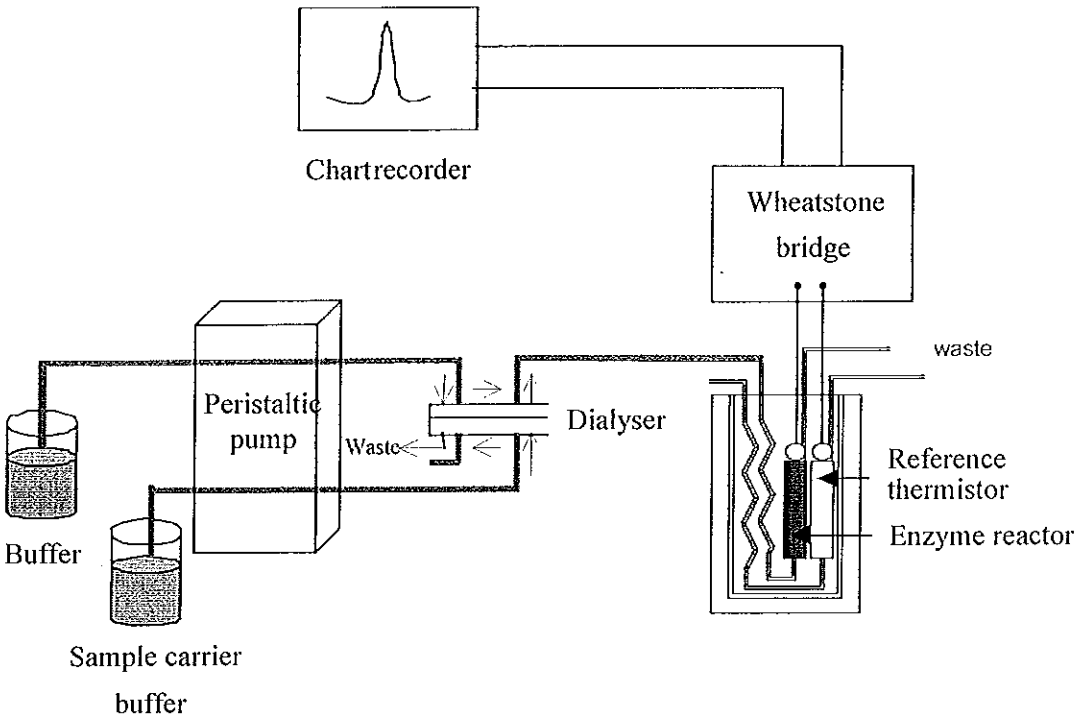


Figure 8 Schematic diagram showing the calorimetric system.

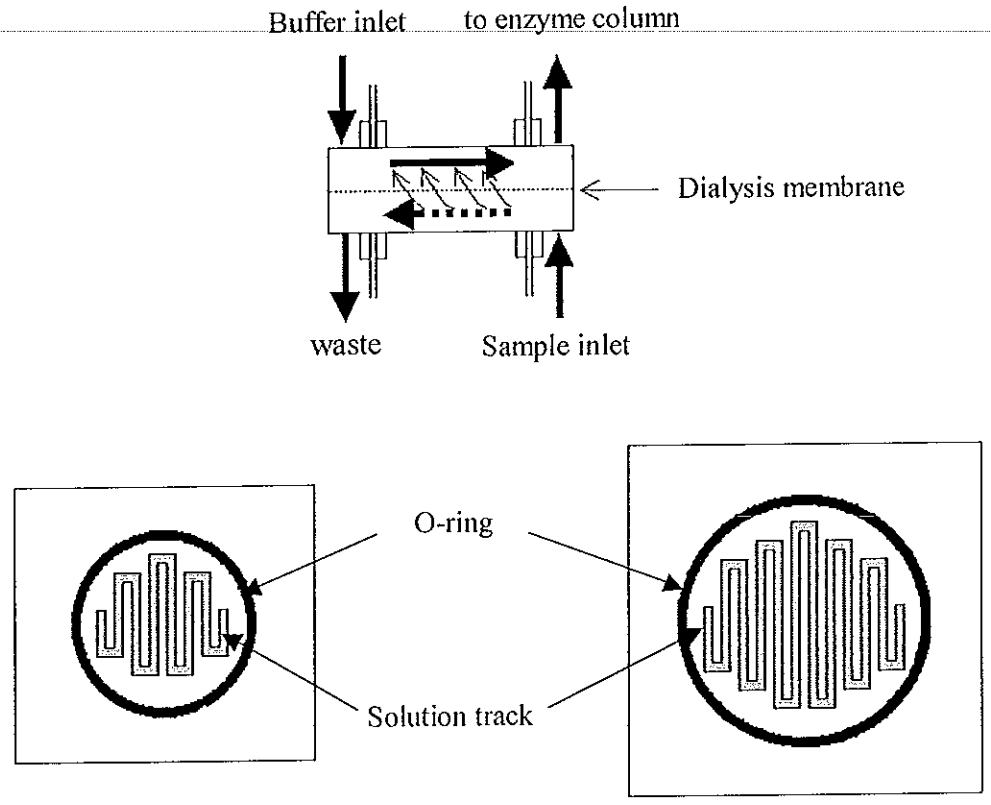


Figure 9 Schematic diagram showing two dialysers with different diffusion area a) 1.5x298.0 mm^2 (amperometric system) and b) 1.5x625.0 mm^2 (calorimetric system)

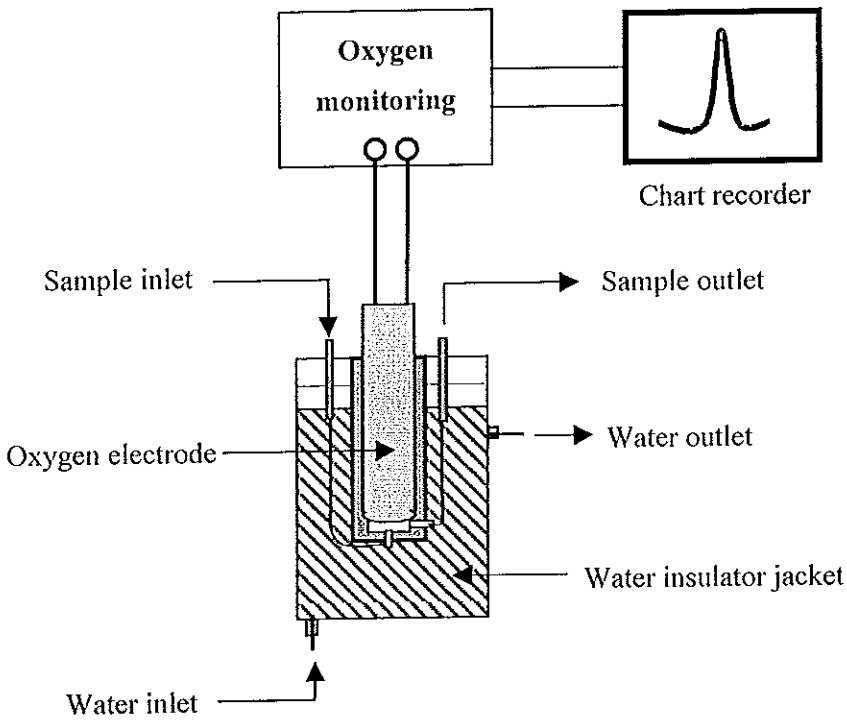


Figure 10 Schematic diagram of the amperometric detection unit.

2.8 Data analysis

The main aim of this work was to compare the efficiency of two transducers, that is oxygen electrode and thermistor, to determine glucose concentration. This was done by testing the responses of immobilized enzyme in these two biosensor systems. The responses were the measured peak heights. The considered parameters were sensitivity, linear range, precision, limit of detection and dynamic characteristic of the response.

Sensitivity is defined as the slope of the calibration graph, provided the plot is linear (Miller and Miller, 1993).

Linear range is the concentration range of analyte giving the linear relation with the signal. Curvilinear regression method (Miller and Miller, 1993) was used in this work.

Precision is shown in term of the standard deviation, %RSD.

Limit of detection is the smallest concentration which gives a quantitative signal. A commonly used definition in the literature of analytical chemistry is the analyte concentration giving signal equal to the blank signal plus two standard deviation of the blank (Miller and Miller, 1993).

Dynamic characteristics of the response are defined as analysis time, peak height, peak width *etc.*

A typical recorder output has the form of a peak (Figure 11 (a)), the height (H), width (W), or area (A) of which is related to the concentration of the analyte. The time span between the sample injection and the peak maximum (S), which yields the analytical readout as peak height, is the residence time. A well-designed flow system has an extremely rapid response. The time used from peak height until the response comes to baseline is washout time and the time used between injection of sample and a 95% decrease of the flow response, is the analysis time (Jurkewicz *et al.*, 1998). In this work we choose peak height to interpret raw data since it is easily identified and directly

related to the detector response. The peak height value (H) is measured as the distance from baseline to peak maximum (Figure 11 (a)). Baseline drift can be corrected for by interpolating the baseline between the start and finish of the peak (Figure 11 (b)) (Snyder and Kirkland, 1979). For each concentration the average of three pulses was obtained.

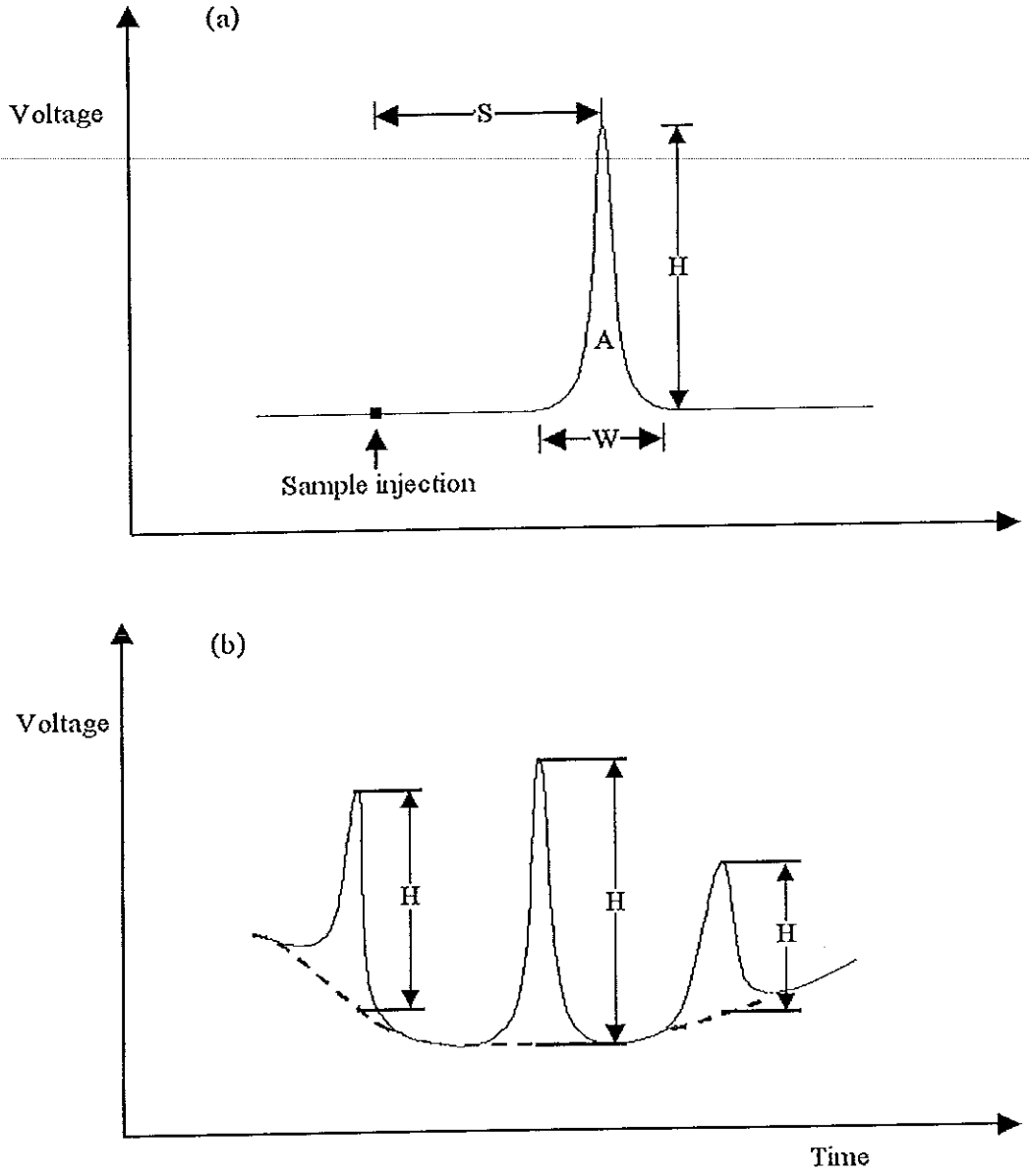


Figure11 Flow injection signals: (a) A typical response of immobilized enzyme to glucose measured as a direct current voltage signal that is related to the oxygen concentration and the heat of the solution as recorded by the analytical system. (S - residence time, A - peak area, H - peak height and W - peak width) (b) Peak-height measurement.

2.9 Optimization

The aim of this part was to optimize the operating conditions of the amperometric and calorimetric biosensors in a flow through and a flow injection analysis systems using enzyme immobilized on silica gel. The optimized variables included flow rate, type, pH and concentration of buffer, and sample volume. These are summarized in Table 1.

All parameters of the flow through and flow injection analysis systems were optimized to obtain a high sensitivity and the shortest period of time. To optimize the factors that may effect the result, the uniparameter variation was used. This was done by changing a single variable while the other parameters are constant. More advanced optimization methods were not applied, as the system was not complex and the methods used was simple, fast and adequate (Jarkiewicz *et al.*, 1998).

Table 1 Assayed and optimized values of the conditions under study for each of the analytical biosensor systems (GOD-Glucose oxidase. CAT-Catalase, NA – not applicable)

Conditions	Transducer		
	Amperometric	Calorimetric	
	GOD	GOD	GOD+CAT
Flow-through system 1.Temperature (°C) at flow cell	20, 30, 50	NA	
2.Flow rate without dialyzer (ml/min)	0.25, 0.50, 0.75, 1.00, 1.25		
3.Flow rate of sample line (ml/min)	0.20, 0.30, 0.40, 0.50, 0.60		
4.Flow rate of buffer line (ml/min)	0.30, 0.50, 0.75, 1.00	0.4, 0.5, 0.75, 1.00, 1.25	
5.Type of buffer	Tris-HCl, EDTA, phosphate buffer	Tris-HCl, EDTA, K_2HPO_4-NaOH, phosphate buffer	
6.Concentration of buffer (M)	0.05, 0.075, 0.10, 0.15, 0.20, 0.25, 0.30	0.01, 0.05, 0.075, 0.10, 0.15, 0.20	
7.pH of buffer	6.00, 6.50, 7.00, 7.20, 7.40, 7.60, 8.00	6.00, 6.50,6.80, 7.00, 7.20, 7.50, 8.00	
8.Sample volume (µl)	200, 300,400, 500, 600		
Flow-injection system 9.Flow rate of sample line (ml/min)	0.20, 0.30, 0.40, 0.50, 0.60		
10.Flow rate of buffer line (ml/min)	0.30, 0.50, 0.75, 1.00	0.40, 0.50, 0.75, 1.00	
11.Sample volume (µl)	200, 300, 400, 500		

2.10 Optimization of the operating conditions in amperometric system

Glucose oxidase is highly specific to β-(D)- Glucose. An aqueous solution of glucose contains about one third of ∝-(D)- Glucose and two third of β-(D)- Glucose in equilibrium. For this reason, it is important to let glucose standard solution stand overnight to allow it to reach equilibrium. As β-(D)- Glucose is oxidized by glucose oxidase, ∝-(D)- Glucose is converted to the β-form (Hansen,1993).

In these studies standard glucose solution (0.1, 0.3, 0.5, 0.7 and 1.0 mM) were prepared in 0.05 M phosphate buffer pH 7.00. A 500 µl sample solution was introduced into the continous flow (0.50 ml/min) of 0.05M phosphate buffer pH 7.00. When the solution containing glucose passed through the column reactor containing immobilized GOD, the amount of oxygen decreased due to the oxidation reaction of glucose catalyed by GOD. The reduction of oxygen in the solution was detected by the oxygen electrode, and the change was recorded on a chart recorder.

2.10.1 The flow through system

2.10.1.1 Optimization of the temperature

In this system the response of the oxygen probe is highly dependent on the temperature, and in particular, the temperature of the membrane adjacent to the cathode where the rate of oxygen consumption by the probe is direct proportion to the current produced by the electrode. Moreover, the permeability of oxygen through the membrane is temperature sensitive (Manual of oxygen monitoring). So the optimization of the water temperature inside the insulator jacket is necessary. This was done by flowing the water at a controlled temperature through the water insulator jacket to control the temperature in the oxygen electrode flow cell chamber (Figure 10). The effect of the temperature was studies at 20, 30 and 50 °C. The sample

volume of standard glucose solution (0.1, 0.3, 0.5, 0.7 and 1.0 mM prepared in 0.05 M phosphate buffer pH 7.00) was 500 µl.

2.10.1.2 Optimization of the flow rates

In a flow system, the flow rate of the solution passing through the reactor and the detector is the main effect of the dispersion of the analyte particles, yield of the reaction and response of the detector (Valcarcel and Luque de castro, 1987). The dispersions that occur will affect the result of the flow system. This is especially true with longitudinal dispersion in the liquid system that causes the decrease of the peak height and the increase of the peak width. An increasing flow rate can decrease the dispersion effects. On the other hand, the yield of the reaction and the response of the detector depend on the retention time of the sample in the reactor and electrode respectively. Thus, it is necessary to find the optimum flow rate.

In this proposed system a dialyser with a diffusion area of 1.5x298.0 mm^2 (Figure 8 (a)) was used and this consisted of two flow lines. A sample line where sample was introduced and a buffer line which carried the analyte that passed through the dialysis membrane from the sample, to the reactor. The flow rate of these two lines would also affect the diffusion of the sample molecules through the membrane and hence the dispersion of the analyte particles. Therefore, we must optimize both. This was done in three steps

Step 1 optimized the flow rate of the system without a dialyser

Step 2 incorporated the dialyser into the system and optimized the flow rate of the sample line while the flow rate of the buffer line was fixed at the optimum as found in step 1

Step 3 fixed the flow rate of the sample line as found in step 2 and re-optimized the flow rate of the buffer line

a) Flow rate without a dialyser

The effect of the flow rate was studied at 0.25, 0.50, 0.75, 1.00 and 1.25 ml min^{-1}. The sample volume of standard glucose solution (0.1, 0.3, 0.5, 0.7 and 1.0 mM prepared in 0.05 M phosphate buffer pH 7.00) was 500 µl.

b) Flow rate of the sample line

The effect of the flow rate of the sample line was studied at 0.20, 0.30, 0.40, 0.50, and 0.60 ml min^{-1}. The flow rate of the buffer line was fixed at 0.75 ml min^{-1} which was the optimum flow rate without a dialyser found in 2.10.1.2.a. The sample volume of the standard glucose solution (0.1, 0.3, 0.5, 0.7 and 1.0 mM prepared in 0.05 M phosphate buffer pH 7.00) was 500 µl.

c) Flow rate of the buffer line

The flow rates of the buffer line were investigated at 0.30, 0.50, 0.75 and 1.00 ml min^{-1}. The flow rate of the sample line was 0.30 ml min^{-1}, i.e. the optimum found in 2.10.1.2.b.

2.10.1.3 Type of buffer solution

The oxidation reaction of glucose occurs when there are oxygen molecules in the solution. Also the sensitivity in oxygen electrode detection is controlled by the dissolved oxygen in the buffer solution. However, the dissolved oxygen in the various buffer solutions are different (Frieder *et al.*, 1987). Therefore a suitable type of buffer should be found. Three types of buffer were tested, that is, Tris-HCl, EDTA and phosphate at 0.05 M and pH 7.00. The sample volume of standard glucose solution (0.1, 0.3, 0.5, 0.7 and 1.0 mM prepared in each type of buffer solution) was 500 µl. The flow rate of the sample line and buffer line were 0.30 and 0.75 ml min^{-1} respectively (optimum flow rates from 2.10.1.2).

2.10.1.4 Buffer concentration

The effect of buffer concentration (viscosity) in an amperometric system has to be examined because viscosity of buffer solution would influence the hydrodynamic flow of fluid permeation rate through the dialysis membrane. The response of the low buffer concentration (low viscosity) was more than that of the high buffer concentration (high viscosity), because the fluid had high permeation rate through the dialysis membrane (www.spectrapore.com), but its handicap was the low buffer capacity. Therefore, the responses of the system to different concentrations were investigated. These were done by using 0.05, 0.075, 0.10, 0.15, 0.20 and 0.30 M phosphate buffer pH 7.00 (best type of buffer solution in 2.10.1.3). Concentrations of standard glucose solution were 0.1, 0.3, 0.5, 0.7 and 1.0 mM (prepared in each concentration of phosphate buffer pH 7.00). The sample volume was 500 µl.

2.8.1.5 pH of the buffer solution

Enzyme activity is known to be depended on pH. The optimum pH for glucose oxidase is about 7.50 and is coincided closely with the pH of blood (Wilson and Turner, 1992). However, when the enzyme is immobilized, the optimal pH may shift, depending on the nature of the support material (Cabral and Kennedy 1991; Guilbault, 1984). Thus, it is necessary to find the optimum pH of buffer solution. This was investigated by using 0.10 M phosphate buffer (optimum buffer concentration in 2.10.1.4.) pH 6.00, 6.50, 7.00, 7.20, 7.40, 7.60 and 8.00. The sample volume of standard glucose solution (0.1, 0.3, 0.5, 0.7 and 1.0 mM prepared in each pH of 0.10 M phosphate buffer) was 500 µl.

2.10.1.6 Effect of 0.9% (w/v) NaCl

The liquid portion of blood is the blood plasma where the usual concentration of salt is isotonic to 0.9% (w/v) NaCl solution (Bloomfeild *et al.,* 1996). The salts have some effect on the enzyme since it has been

reported that some ion such as Na^+ and K^+ are inhibitors of enzyme. This possible effect was investigated by comparing the responses from three cases

I) Buffer line, sample line and standard solution were without NaCl.

II) Buffer line and sample line were without NaCl. Standard solution was with 0.9 %(w/v) NaCl.

III) Buffer line was without NaCl. Sample line and standard solution were with 0.9% (w/v) NaCl.

The sample volume was 500 µl. Concentrations of standard glucose solution were 0.1, 0.3, 0.5, 0.7 and 1.0 mM prepared in 0.10 M phosphate buffer pH 7.20 (optimum pH of buffer solution in 2.10.1.5.).

2.10.1.7 Sample volume

One way of improving the response of the system is to increase analyte by increasing the sample volume. However, in an enzymatic analysis the reaction yield also depends on the amount of enzyme. So, too much of the analyte for the same amount of enzyme can not increase the response. Moreover, large sample volume may increase the particle dispersion. Therefore, a suitable sample volume should be found. The tested volumes were 200, 300, 400, 500 and 600 µl. Concentrations of standard glucose solution were 0.1, 0.3, 0.5, 0.7 and 1.0 mM (prepared in 0.10 M phosphate buffer pH 7.20 with 0.9% (w/v) NaCl). The flow rate of the sample line (0.10 M phosphate buffer pH 7.20 with 0.9% (w/v) NaCl) was 0.30 ml min^{-1}. The flow rate of the buffer line (0.10 M phosphate buffer pH 7.20 without 0.9% (w/v) NaCl) was 0.75 ml min^{-1} (optimum in 2.10.1.6.(c)).

2.10.2 The flow injection system

The biosensor system develop in this work was aiming to improve the precision and sensitivity. In these studies the concentrations of glucose tested were 0.1, 0.3, 0.5, 0.7 and 1.0 mM prepared in 0.10 M phosphate buffer pH 7.00 with contain 0.9% (w/v) NaCl.

2.10.2.1 Optimization of the flow rates

a) Flow rate of the sample line

The flow rates of the sample were studied at 0.20, 0.30, 0.40, 0.50 and 0.60 ml min^{-1}. The flow rate of the buffer line was fixed at 0.75 ml min^{-1}. The sample volume was 500 µl.

b) Flow rate of the buffer line

The flow rate of buffer line was investigated at 0.25, 0.50, 0.75 and 1.00 ml min^{-1}. The flow rate of sample line was 0.30 ml min^{-1} which was the optimum flow rate of the sample line found in 2.10.2.1.a). The sample volume was 500 µl.

2.10.2.2 Sample volume

The effect of the sample volume was determined. The tested volume were 200, 300, 400 and 500 µl. The flow rates of the sample and buffer lines were 0.30 and 0.75 ml min^{-1} respectively. These are the optimum flow rates found in 2.10.2.1.

2.10.3 Comparison of the flow through and flow injection analysis

The difference between the flow through and flow injection were compared at their optimum conditions. The parameters considered were sensitivity, limit of detection, precision and dynamic characteristic of the response.

2.10.4 Stability of the enzyme reactor column

The long-term stability of the immobilized glucose oxidase activity on silica gel was tested intermittently over a period of 5 months by monitoring its response to glucose standard solutions. Sensitivity and precision of the calibration curve were used as indicating factors of the column reactor.

2.10.5 Reproducibility of immobilized enzyme

In an enzyme-based biosensor system, the responses would decline with time due to the degradation of the enzyme. Although a new reactor can be changed easily, its response should be the same as the old one so the analysis can be carried on. In this part the reproducibility of the activity of different enzyme reactors was investigated. The responses of two enzyme reactor columns where the enzyme was immobilized in the same batch were compared. In another experiment the response of immobilized glucose oxidase from different batches were investigated. These studies were done by monitoring the calibration curve of 500 µl of standard glucose solutions (0.1 to 1.0 mM).

2.10.6 Linearity of the responses of glucose oxidase

The linearity of the response of glucose oxidase was investigated under the optimum condition in the flow injection system *i.e.* sample volume 500 µl, flow rates of buffer and sample lines 0.3 and 0.75 ml min^{-1} respectively.

2.11 Optimization of the operating conditions in Calorimetric system

Calorimetric biosensor system using the enzyme reactor column was optimized (Table 1). In this system two different enzyme reactor columns were tested. One column contained glucose oxidase co-immobilized with catalase and the other only had immobilized glucose oxidase. Both enzyme reactor columns were optimized using the same operating conditions. The standard glucose solution was prepared as in the amperometric system.

2.11.1 The flow through system

2.11.1.1 Optimization of the flow rates

a) Flow rate without a dialyser

The effect of the flow rate was studied 0.25, 0.50,

0.75, 1.00 and 1.25 ml min^{-1}. The sample volume of standard glucose solution 0.1, 0.3, 0.5, 0.7, and 1.00 mM prepared in 0.05 M phosphate buffer pH 7.00, was 500 µl.

b) Flow rate of the sample line with a dialyser

The flow rate of the sample line was investigated at 0.20, 0.30, 0.40, 0.50 and 0.60 ml min^{-1} by fixing the flow rate of the buffer line at its optimum (2.11.1.1.a), 0.75 ml min^{-1}. The sample volume of the standard glucose (3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 mM) was 500 µl.

c) Flow rate of the buffer line with a dialyser

The flow rate of buffer line were studied at 0.25, 0.50, 0.75, 1.00 and 1.25 ml min^{-1}. The flow rate of the sample line was 0.40 ml min^{-1} (optimum flow rate in 2.11.1.1.b).

2.11.1.2 Type of buffer solution

The effect of type of buffer solution in a calorimetric system has to be examined because the deprotonation reaction of the buffer solution will occur as soon as the buffer solution absorbs (endothermic reaction) or emits (exothermic reaction) heat. Since the oxidation reaction of glucose is an exothermic reaction, the heat will be released into the buffer. If the deprotonation reactions of the buffer used is endothermic the net heat will be absorbed and this will reduce the sensitivity of the system (Bataillard *et al.*, 1993; Bjarnason *et al.,* 1998). Therefore, the response of the system to different type of buffer solution was studied.

The effect of the type of buffer solution was studied using four types of buffer, that is, Tris-HCl, EDTA, K_2HPO_4-NaOH and phosphate at 0.05 M and pH 7.00. The sample volume of standard glucose solution (3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 mM prepared in each type of buffer solution) was 500 µl. The flow rate of the sample line and buffer line were 0.40 and 0.75 ml min^{-1} respectively (optimum flow rate of the buffer line in 2.11.1.1).

2.11.1.3 Buffer concentration

The buffer concentration was investigated at 0.01, 0.05, 0.075, 0.10, 0.15 and 0.20 mM of phosphate buffer (optimum type of buffer in 2.11.1.2). Concentration of standard glucose solution was 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 mM. The sample volume was 500 µl.

2.11.1.4 pH of buffer solution

The effect pH of buffer solution were studied at 6.00, 6.50, 6.80, 7.00, 7.20, 7.50 and 8.00 of 0.1 M phosphate buffer (optimum buffer concentration in 2.11.1.3) Concentration of standard glucose were 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 mM. The sample volume was 500 µl.

2.11.1.5 Effect of 0.9% (w/v) NaCl

The effect of 0.9% (w/v) NaCl was investigated as in the amperometric system (2.8.1.6). The sample volume was 500 µl. Concentration of standard glucose solution were 0.1, 0.3, 0.5, 0.7 and 1.0 mM.

2.11.1.6 Sample volume

The effect of the sample volume was determined. The tested volume were 200 to 600 µl. Concentration of standard glucose were 0.1, 0.3, 0.5, 0.7 and 1.0 mM prepared in 0.10 M phosphate buffer pH 7.00 with 0.9% (w/v) NaCl (optimum in 2.9.1.5)

2.11.2 The flow injection system

2.11.2.1 Optimization of the flow rates

a) The flow rate of the sample line

The flow rate of the sample were studies at 0.20, 0.30,0.40,0.50 and 0.60 ml min^{-1}. The flow rate of the buffer line was fixed at 0.75 ml min^{-1}. The sample volume was 500 µl.

b) Flow rate of the buffer line

The flow rate of buffer line was investigated at 0.40 to 1.00 ml min^{-1}. The flow rate of sample line was 0.40 ml min^{-1} which

was the optimum flow rate of the sample line found in 2.11.2.1.a). The sample volume was 500 µl.

2.11.2.2 Sample volume

The effect of the sample volume was determined. The tested volume were 200, 300, 400 and 500 µl. The flow rates of the sample and buffer lines were 0.40 and 0.75 ml min^{-1} respectively. These are the optimum flow rates found in 2.112.1.

2.11.3 Comparison of response of calorimetric in the flow through and flow injection analysis.

The system of calorimetric biosensers in the flow-through and flow-injection system of both enzyme reactor column were compared at their optimum conditions by considering sensitivity and precision which then was used to determine glucose concentration in the human plasma samples. Concentration of standard glucose solution were prepared in 0.10 M phosphate buffer pH 7.0 The sample volume was 500 µl (optimum sample volume in 2.11.2.2).

2.11.4 Stability of the enzyme reactor column

To test the stability of the enzyme reactor column the same procedures as in the amperometric system were followed. The flow rates of buffer and sample lines were 0.4 and 0.75 ml min^{-1} respectively. The buffer used was 0.1 M phosphate buffer pH 7.0.

2.11.5 Linearity of the responses of enzyme

The linearity of the response of both enzyme reactor columns was investigated under the optimum condition in the flow injection system *i.e.* sample volume 500 µl, flow rate of buffer and sample lines 0.4 and 0.75 ml min^{-1} respectively.

2.12 Determination of glucose in the plasma samples

To demonstrate the use of the amperometric and calorimetric biosensors the systems were tested using the plasma samples obtained from Songklanagarind Hospital, Prince of Songkla University. The same sample was analyzed by UV-visible spectrophotometry using the commercially glucose kit and the hexokinase method (the results obtained by Songklanagarind Hospital).

2.12.1 Amperometric biosensor

Before glucose was measured, the plasma samples were diluted using 0.10 M phosphate buffer pH 7.20 (contained 0.9 %(w/v) NaCl) at a plasma : buffer ratio of 1:9.

To calibrate the system, 500 µl standard glucose solutions (0.1, 0.5, 1.0, 2.0, 4.0 and 6.0 mM prepared in 0.10 M phosphate buffer pH 7.20+0.9% (w/v) NaCl) were injected into the system. The flow injection system was chosen because of its high sensitivity and precision. The flow rates of the buffer line was 0.75ml min^{-1} and sample lines was 0.30 ml min^{-1}. The calibration curve was prepared by plotting the amperometric response *versus* corresponding glucose concentration (mM). The sample solutions were then injected into the system. The change in the oxygen of each sample was used to calculate the glucose concentration from the calibration done prior to the test.

2.12.2 Calorimetric biosensor

The plasma samples were diluted using 0.10 M phosphate buffer pH 7.0 (contained 0.9% (w/v) NaCl) at a plasma : buffer ratio of 1:9.

To calibrate the system, standard glucose solutions (0.3-8.0 mM prepared in 0.10 M phosphate buffer pH 7.0+0.9% (w/v) NaCl) were injected into the system. In this system was chosen flow injection system to determine glucose concentration in plasma samples. The flow rate of the buffer line was

0.75 ml min^{-1} and sample line was 0.40 ml min^{-1} and the sample volume was 500 µl. The calibration curve was prepared by plotting the calorimetric response *Vs* corresponding glucose concentration (mM). The sample solutions were then injected into the system. The change in the temperature of each sample was used to calculate the glucose concentration from the calibration done prior to the test.

2.12.3 Hexokinase method (Songklanagarind Hospital)

The plasma samples were tested by Songklanagarind Hospital using an autoanalyzer (Hitachi, model 917). This system uses the enzyme hexokinase (HK) which catalyzes the phosphorylation of glucose to glucose-6-phosphate (G-6-P) by ATP. Then glucose-6-phosphate dehydrogenase (G-6-PD) oxidizes glucose-6-phosphate in the present of $NADP^+$ to gluconate-6-phosphate.

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + NADP^+ \xrightarrow{\text{G-6-PD}} \text{gluconate-6-phosphate} + NADPH + H^+$$

The rate of $NADP^+$ formation during the reaction is directly proportional to the glucose concentration and can be measured photometrically (Hanson, 1993).

2.12.4 Glucose Sigma Kit

The Sigma procedure is based on the following enzymatic reactions:

$$\text{Glucose} + H_2O_2 + O_2 \xrightarrow{\text{GOD}} \text{Gluconolactone} + H_2O_2$$

$$H_2O_2 + \text{o-Dianisidine} \xrightarrow{\text{POD}} \text{Oxidized o-Dianisidine}$$

(colorless) (Brown)

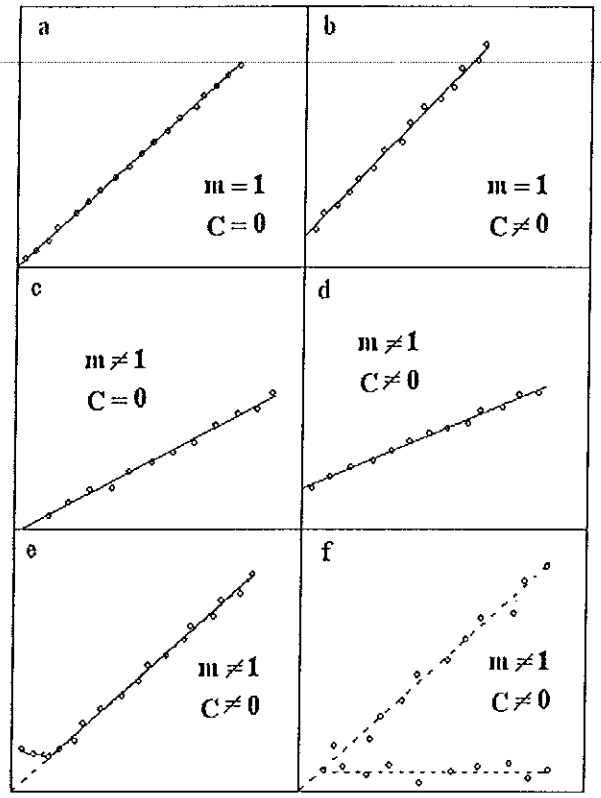
The intensity of the brown color was measured at 425-475 nm using a spectrophotometer.

2.13 Comparison of the results

The amperometric and calorimetric systems were validated by comparing the results to those of the enzymatic reaction (result from Songklanagarind Hospital and Glucose Sigma Kit). In making such a comparison, the principle interest will be whether the proposed method gives results that are significantly higher or lower than the established methods. So, the analysis using the regression line (Miller and Miller, 1993) and the Wilcoxon signed rank test (Freund and Simon, 1997; Miller and Miller, 1993; Triola, 1998) were used in this work.

2.13.1 Regression line analysis

The regression line (Figure 12) can be used to compare two methods by plotting one axis of the regression graph using the results obtained by the proposed method and the other axis the results obtained from the comparison method of the same samples. Each point on the graph, thus, represents a single sample analyzed by two separate methods. The slope (m), the intercept (C), and the production moment correlation coefficient (r^2) of the regression line are then calculated. It is clear that if each sample yields an identical result with both analytical methods the regression line will have zero intercept and a slope and a correlation coefficient of 1 (Figure 12 (a)). In practice this never occurs even if systematic errors are entirely absent, random errors ensure that the two analytical procedures will not give results in exact agreement for all the samples (Figure 12 (b–f)). The most common tests to be done is to test whether an intercept (C) differs significantly from zero, and a slope (m) differs significantly from 1. Determining the errors in the slope (Sm) and intercept (Sc) of the regression line at 95 % significant level performs such tests. If $m \pm S_m$ cover 1 and $C \pm S_c$ cover zero, there are no systematic errors and the result are then accepted (Miller and Miller, 1993).



$Y = mX + C$

$r^2 =$ .....

Figure 12 The use of a regression line to compare two analytical methods;

(a) shows perfect agreement between the two methods for all the samples; (b)–(f) illustrate the result of various type of systematic errors of the slope and / or the interception.

(Redrawn form Miller and Miller, 1993)

2.13.2 Wilcoxon signed rank test

The Wilcoxon signed rank test is one type of statistical tests uses to handle data which may not be normally distributed. The null hypothesis in these case is 'there is no systematic difference between the two methods'. The comparison was done by comparing each pair of data. Then these values were arranged in numerical order without regard to sign. The numbers were then ranked; in this process they keep their signs but are assigned number indicating their order. The positive and negative ranks were summed individually. The test statistic is either the sum of the positive or negative ranks depended on the alternative hypothesis (see later). If the test statistic is less than the critical value given in Table 2 ($P<0.05$) then the null hypothesis is reject.

2.13.2.1 Two-tailed test

The two-tailed test was applied first to test whether there was any significant difference between the two methods. In this case the alternative hypothesis is 'there is significant difference between the two methods', *i.e* method 1 is either more than or less than method 2. The test statistic is the lower rank sum of the positive or negative rank. The null hypothesis is rejected if the test statistic is less than or equal to the tabulated value in Table2.

2.13.2.2 One-tailed test

When the comparison of any two methods failed the two-tailed Wilcoxon signed rank test or the regression line test, the one-tailed Wilcoxon singed rank test is used to find out whether the differences between the methods are systematic. The test is used to test the same null hypothesis that there is no difference between the two methods while an alternative hypothesis is that one method gives higher, or lower value than the other method. The test statistic, the sum of negative ranks is used when the proposed method is higher than the reference method. If the test statistic is less than or equal to the tabulated value in Table 2, the null hypothesis is rejected, that is,

the proposed method is higher than the reference method. In contrast, the test statistic is the sum of the positive ranks when the proposed method is lower than the other method.

Table 2 Critical values for the Wilcoxon signed rank test; statistic at $P < 0.05$ for n = 7 to 50 where n is the number of data pair (Triola, 1998). The null hypothesis can be rejected when the test statistic is ≤ the tabulated value.

n	One-tailed	Two-tailed	n	One-tailed	Two-tailed
7	4	2	29	141	127
8	6	4	30	152	137
9	8	6	31	163	148
10	11	8	32	175	159
11	14	11	33	188	171
12	17	14	34	201	183
13	21	17	35	214	195
14	26	21	36	228	208
15	30	25	37	242	222
16	36	30	38	256	235
17	41	35	39	271	250
18	47	40	40	287	264
19	54	46	41	303	279
20	60	52	42	319	295
21	68	59	43	336	311
22	75	66	44	353	327
23	83	73	45	371	344
24	92	81	46	389	361
25	101	90	47	408	379
26	110	98	48	427	397
27	120	107	49	446	415
28	130	117	50	466	434

Chapter 3

Results and Discussion

3.1 Characteristics of the biosensor response

Typical signal responses of immobilized enzyme in the amperometric and calorimetric systems are shown in Figures 13 and 14, respectively. The amplitude of the signal (H) which is directly related to glucose concentration was measured. The response time and analysis time were also considered.

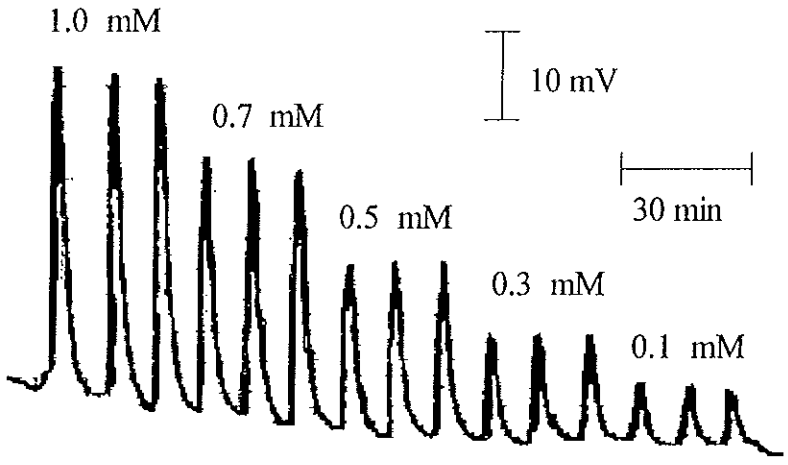


Figure 13 Responses of glucose oxidase in the amperometric biosensor system using 500µl of glucose and 0.1M phosphate buffer pH 7.20 at flow rates of buffer and sample lines 0.75 and 0.3 ml min^{-1}, respectively.

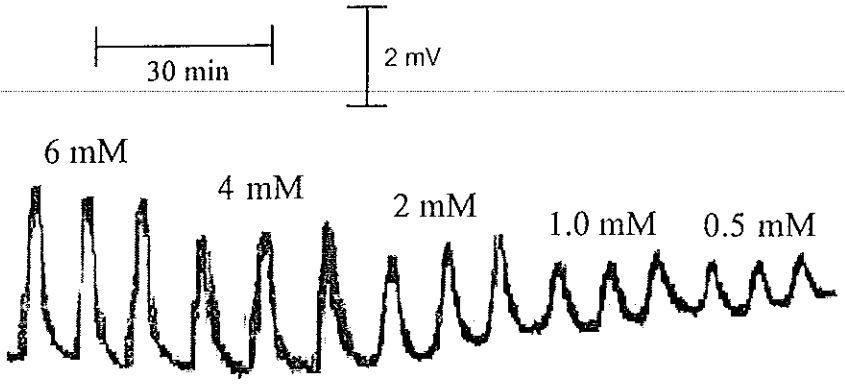


Figure 14 Responses of glucose oxidase and catalase in the calorimetric biosensor system using 500 µl of glucose and 0.1M phosphate buffer pH 7.00 at flow rates of buffer and sample lines 0.75 and 0.4 ml min^{-1}, respectively.

Three signals were obtained for each concentration where the mean and standard deviation (SD) were then calculated. In some cases the three signals were of the same size, *i.e.* SD=0 and the zero value will be shown in the Tables in the sections which followed this. However, it would be best pointed out here that the uncertainty due to the reading of the measuring scale in all cases is 0.5 mm which is equivalent to 0.5 mV for the amperometric system and 0.12 mV for the calorimetric system.

3.2 Amperometric system

3.2.1 The flow through system

3.2.1.1 Optimization of the temperature

By using different water temperature in the insulator jacket different temperature of the solution passing through the flow cell were controlled. The temperature in the flow cell corresponded to 50°C, 30°C and 20°C of the water in the insulator jacket were 27-29°C, 24-26°C and 21-23°C respectively. The responses of the enzyme reactor column to different temperatures of the water insulator jacket are shown in Table 3 and Figure 15. The peak heights and sensitivities increased as the temperature increased. The higher temperature (50°C) gave the highest sensitivity but also difficult to control since it was much different from the room temperature. Therefore, 30 °C was chosen.

Table 3 Responses of the glucose amperometric biosensor system using different temperature at the water insulator jacket

Glucose concentration (mM)	Amperometric response (mV), different temperature at water insulator jacket		
	20°C	**30°C**	50°C
	Mean ± SD	**Mean ± SD**	Mean ± SD
0.1	16.0±0.0	**33.3±2.3**	41.3±2.3
0.3	40.0±0.0	**78.6±2.3**	86.0±4.0
0.5	61.3±2.3	**134±2.3**	150±2.3
0.7	99.3±2.3	**199±2.3**	222±4.0
1.0	136±4.0	**264±4.0**	285±2.3
Sensitivity (mV/mM)	136	**264**	282
R^2	0.9926	**0.9954**	0.9913
Analysis time (min)	8-10	**8-10**	8-10

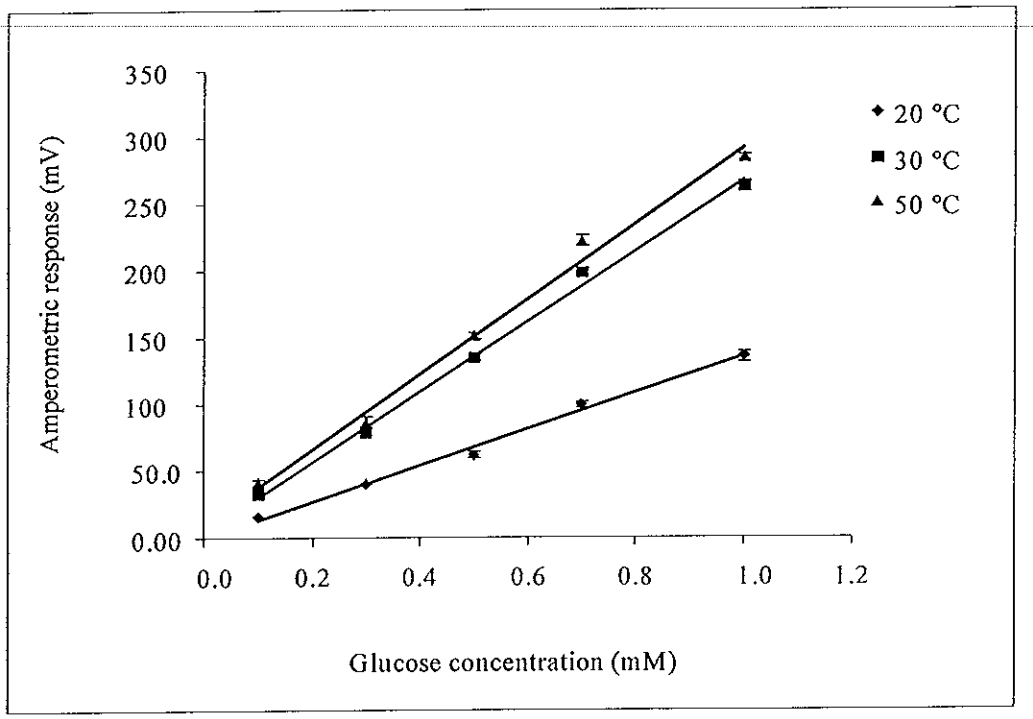


Figure 15 Responses of the glucose amperometric biosensor system at different water temperature in the insulator jacket

3.2.1.2 Optimization of the flow rate

a) Flow rate without a dialyser

The effect of the different flow rate on the response was investigated. The responses of 0.1 to 1 mM of standard glucose at different flow rates are shown in Table 4 and Figure 16. The peak heights and sensitivities increase as the flow rate decreased. This is as expected since the standard glucose solution passing through the reactor at different flow rates will affect the response of enzyme and retention time of analyte in the column reactor. The response at a lower flow rate is higher since the glucose solution is retained longer in the column reactor, the reaction is more completed, so the signal is higher. Flow rates of 0.25 ml min^{-1} and 0.50 ml min^{-1} gave high sensitivity but the analysis times were also long (30-40 min and 20-25 min respectively). In contrast higher flow rates (1.25 ml min^{-1} and 1.00 ml min^{-1}) used shorter analysis time (7-8 min) but the high pressure gradients pressed the immobilized enzyme towards the end of the reactor column, clogging up to flow system. Therefore, the flow rate of 0.75 ml min^{-1} was chosen because it gave relatively high sensitivity and short analysis time without affecting the flow of the system.

Table 4 Responses of the glucose amperometric biosensor system using different flow rate without a dialyser

Glucose concentration (mM)	Amperometric response (mV), different flow rate without dialyser				
	0.25 ml/min	0.50 ml/min	**0.75 ml/min**	1.00 ml/min	1.25 ml/min
	Mean ± SD	Mean ± SD	**Mean ± SD**	Mean ± SD	Mean ± SD
0.1	72.6±3.1	48.0±0.0	**30.6±2.3**	24.0±0.0	8.0±0.0
0.3	160±4.0	106±4.0	**74.6±2.3**	48±4.0	33.3±2.3
0.5	223±1.2	156±2.3	**130±2.3**	87±5.0	54.6±2.3
0.7	282±4.0	224±2.3	**194±2.3**	130±2.3	90.6±2.3
1.0	353±2.3	298±2.3	**257±2.3**	200±2.0	121±2.3
Sensitivity (mV/mM)	308	281	**260**	199	129
R^2	0.9848	0.9976	**0.9948**	0.9884	0.9928
Analysis time (min)	30-40	20-25	**8-10**	7-9	6-8

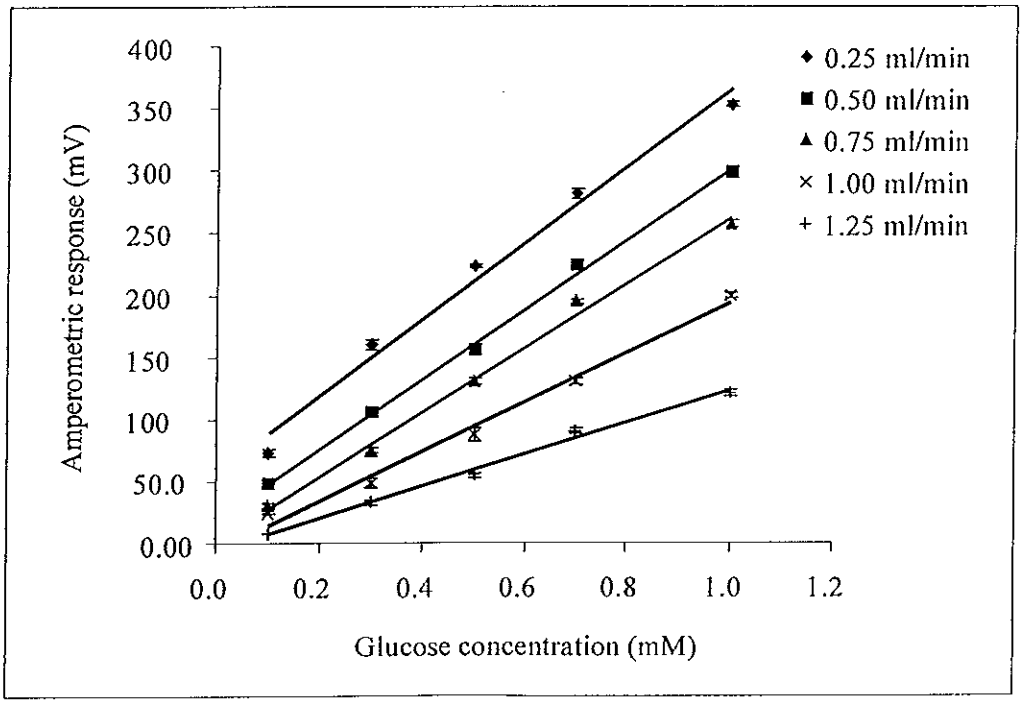


Figure 16 Responses of the glucose amperometric biosensor system using different flow rate without dialyser

b) Flow rate of sample line with a dialyser

To avoid the interference from proteins and other large molecules a dialyser was incorporated into the system. This causes the analyte to be diluted and this depended on the dialyser factor, C_d/C_s. This factor is defined as output concentration, C_d, normalized by division with the initial sample concentration, C_s (Fang, 1993). Therefore, the peak heights of standard glucose concentration are lower when compared with a system without a dialyser.

Flow rate of the sample line directly affects the diffusion efficiency of the standard glucose solution through the dialysis membrane. A slower flow rate allows longer interaction between the analyte and diffusion area of the dialyser, thus the diffusion efficiency is increased resulting in a higher signal (Valcarcel and Luque de castro, 1987). However it also allows the increase of the dispersion phenomenon in the direction of flow and the analyte has more time to be diluted along the flow line. Table 5 and Figure 17 show the results obtained using different flow rates of the sample line. The peak heights and the sensitivities increased as the flow rate decreased . In this case the flow rate of 0.3 ml min^{-1} was chosen because its high sensitivity and the analysis time was much shorter than at 0.20 ml min^{-1} (9 min compared to 12 min)

Table 5 Responses of the glucose amperometric biosensor system using different flow rate of sample line with the flow rate of buffer line 0.75 ml min^{-1}

Glucose concentration (mM)	Amperometric response (mV), different flow rate of sample line				
	0.20 ml/min	**0.30 ml/min**	0.40 ml/min	0.50 ml/min	0.60 ml/min
	Mean ± SD	**Mean ± SD**	Mean ± SD	Mean ± SD	Mean ± SD
0.1	9.5±0.3	**7.9±0.1**	6.1±0.2	5.1±0.5	3.6±0.4
0.3	17.7±0.2	**15.2±0.2**	13.4±0.4	11.2±0.0	10.1±0.1
0.5	24.0±0.2	**22.0±0.3**	20.3±0.2	18.6±0.3	16.3±0.2
0.7	31.6±0.4	**29.3±0.1**	27.6±0.2	25.7±0.4	23.5±0.2
1.0	40.3±0.4	**37.3±0.2**	35.2±0.5	32.6±0.4	30.6±0.4
Sensitivity (mV/mM)	34.1	**33.0**	32.7	31.4	30.4
R^2	0.9963	**0.9956**	0.9943	0.9914	0.9951
Analysis time (min)	12-14	**9-11**	9-8	9-8	7-8

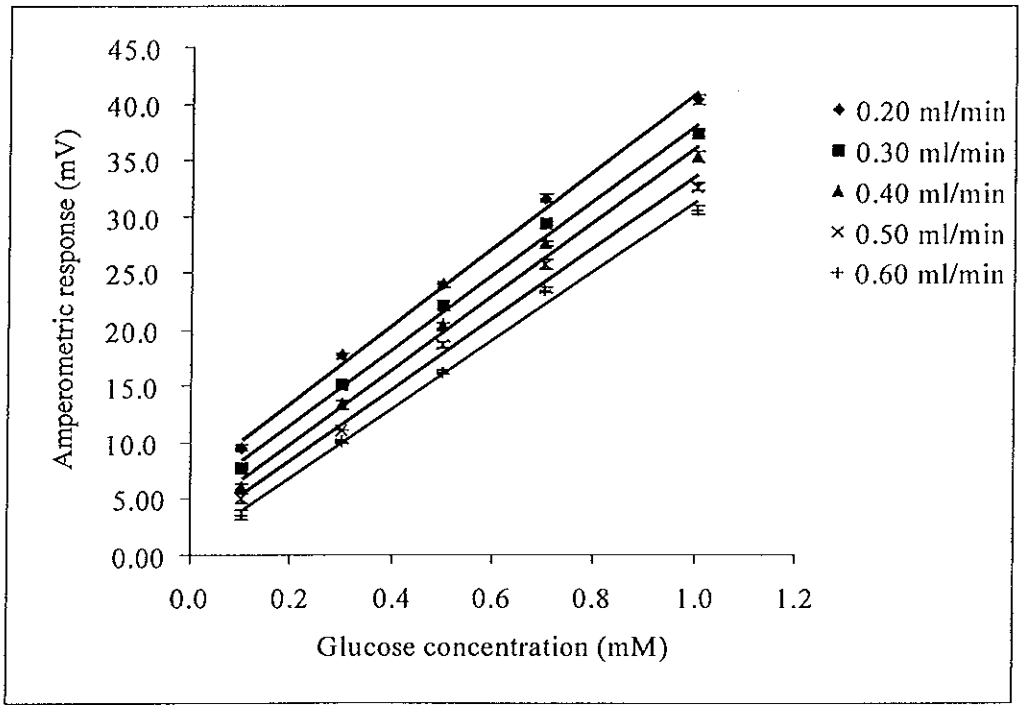


Table 17 Responses of the glucose amperometric biosensor system using different flow rate of sample line

c) Flow rate of the buffer line with a dialyser

The effect of the flow rate of buffer line from 0.30 to 1.00 ml min^{-1} are shown in Table 6 and Figure 18. Higher flow rate caused the peak heights and sensitivities to decrease. When the flow rate of the buffer line increased its pressure decreased (Bernoulli's Equation; Halliday, *et al.*, 1997). Since the flow rate of the sample line was fixed at 0.3 ml min^{-1}. The pressure gradient across the dialysis membrane increased with the buffer flow rate. Therefore, the diffusion efficiency increased resulting in a higher peak height. However, when the flow rate was too fast there would be less time for the enzymatic reaction, thus, a lower response. Therefore, 0.75 ml min^{-1} was chosen, because it gave relatively high signal and sensitivity with less analysis time.

Table 6 Responses of the glucose amperometric biosensor system using different flow rate of buffer line with the flow rate of sample line 0.30 ml min^{-1}

Glucose concentration (mM)	Amperometric response (mV), different flow rate of buffer line			
	0.30 ml/min	0.50 ml/min	**0.75 ml/min**	1.00 ml/min
	Mean ± SD	Mean ± SD	**Mean ± SD**	Mean ± SD
0.1	18.5±0.5	12.6±1.1	**7.7±0.1**	4.8±0.0
0.3	26.3±0.4	20.8±0.7	**14.8±0.3**	10.4 ±0.4
0.5	31.7±0.5	27.3±1.1	**21.3±0.3**	16.4 ±0.2
0.7	39.5±0.5	32.6±1.0	**27.8±0.1**	23.2 ±0.5
1.0	47.8±0.5	41.3±1.1	**35.5±0.3**	30.5 ±0.5
Sensitivity (mV/mM)	32.5	31.2	**31.0**	29.1
R^2	0.9958	0.9929	**0.9955**	0.9970
Analysis time (min)	25-28	20-23	**9-11**	8-10

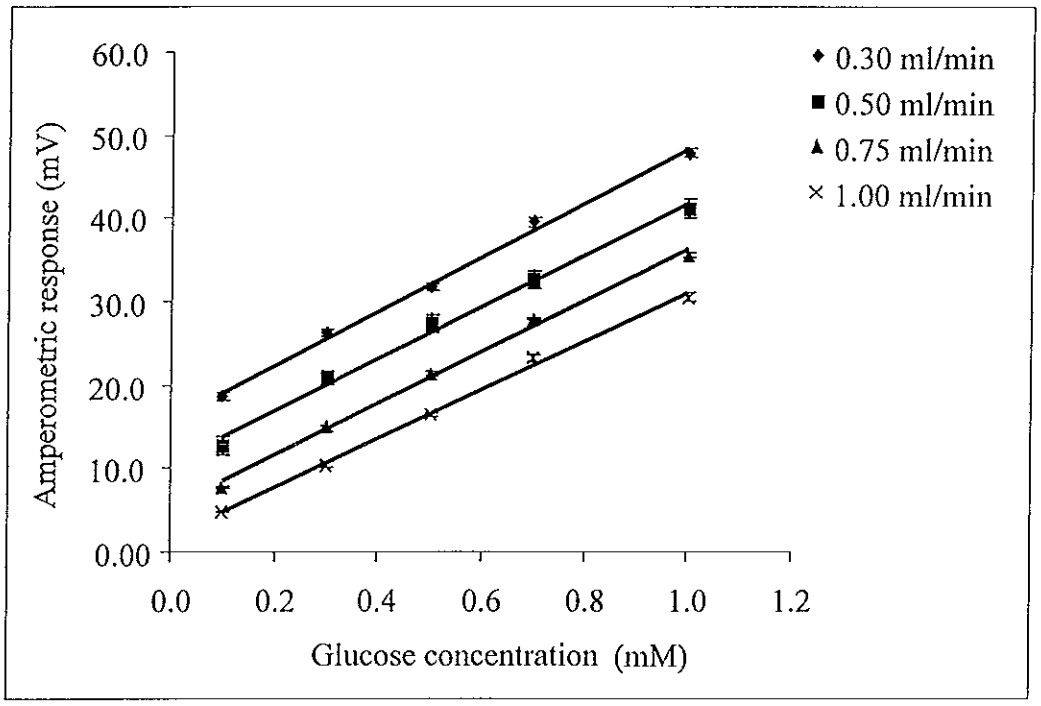


Figure 18 Responses of the glucose amperometric biosensor system using different flow rate of buffer line

3.2.1.3 Type of buffer solution

The effect of three buffer solutions was investigated. The responses of 0.1 to 1.0 mM standard glucose solution in different type of buffer solution are shown in Table 7 and Figure 19. The peak heights and sensitivities when using phosphate buffer solution were higher than those of EDTA and Tris-HCl buffers and phosphate buffer was chosen for further work.

Table 7 Responses of the glucose amperometric biosensor system using different type of buffer solution

Glucose concentration (mM)	Amperometric response (mV), different type of buffer solution		
	Phosphate buffer	Tris-HCl	EDTA-buffer
	Mean ± SD	Mean ± SD	Mean ± SD
0.1	**7.3±0.4**	3.3±0.2	4.7±0.1
0.3	**13.9±0.1**	9.7±0.6	12.3±0.2
0.5	**20.4±0.2**	15.3±1.2	17.8±0.4
0.7	**26.5±0.5**	22.8±0.6	24.2±0.4
1.0	**34.1±0.4**	28.8±1.2	31.6±0.5
Sensitivity (mV/mM)	**29.9**	28.7	29.6
R^2	**0.9963**	0.9905	0.9941
Analysis time (min)	**9-11**	9-11	9-11

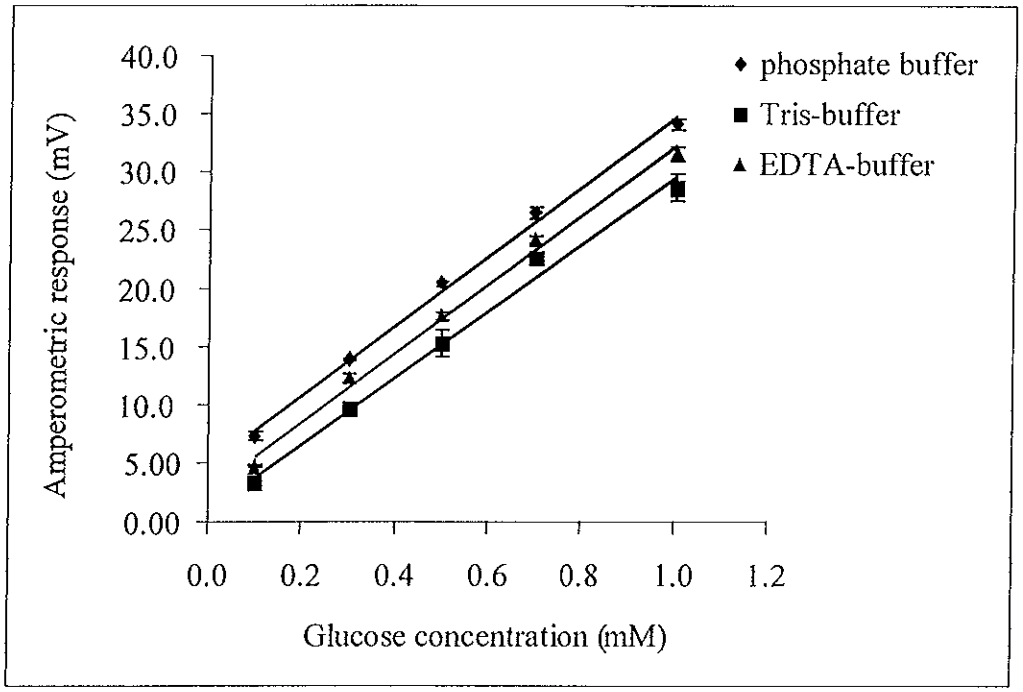


Figure 19 Responses of the glucose amperometric biosensor system using different type of buffer solution

3.2.1.4 Buffer concentration

Table 8 and Figure 20 show the effect of buffer concentration. The response obtained using phosphate buffer pH 7.00 at 0.01, 0.05, 0.075, 0.10, 0.15, 0.20, 0.30 M. Peak heights and sensitivity at 0.1 M phosphate buffer were the highest. This may be because the high concentration (high viscosity fluid) has low permeation rate through the dialysis membrane (www. spectrapore.com) resulting in lower response. At low concentration (low buffer capacity) the stability of buffer solution was rather poor, so, pH might change during the measurement. Therefore, the buffer concentration of 0.10 M phosphate buffer pH 7.00 was chosen.

Table 8 Responses of the glucose amperometric biosensor system using different buffer concentration

Glucose concentration (mM)	Amperometric response (mV), different buffer concentration						
	0.01 M	0.05 M	0.075 M	**0.10 M**	0.15 M	0.20 M	0.30 M
	Mean±SD	Mean±SD	Mean±SD	**Mean±SD**	Mean±SD	Mean±SD	Mean±SD
0.1	4.6±0.5	5.7±0.2	6.4±0.1	**7.0±0.0**	5.9±0.1	5.0±0.0	4.1±0.1
0.3	9.3±0.4	11.5±0.3	12.3±0.3	**12.8±0.2**	11.4±0.4	10.3±0.4	8.9±0.1
0.5	13.5±0.5	16.3±0.4	17.9±0.4	**19.0±0.2**	16.5±0.4	14.6±0.4	12.7±0.4
0.7	18.3±0.6	20.9±0.3	22.2±0.2	**25.1±0.4**	21.5±0.5	19.5±0.4	16.5±0.5
1.0	24.0±1.0	27.5±0.5	30.4±0.4	**32.6±0.5**	28.7±0.6	25.4±0.5	20.8±0.6
Sensitivity (mV/mM)	21.6	24.0	26.3	**28.8**	25.2	22.6	18.4
R^2	0.9981	0.9970	0.9980	**0.9980**	0.9993	0.9972	0.9889
Analysis time(min)	9-10	9-11	9-11	**9-11**	9-11	10-12	10-12

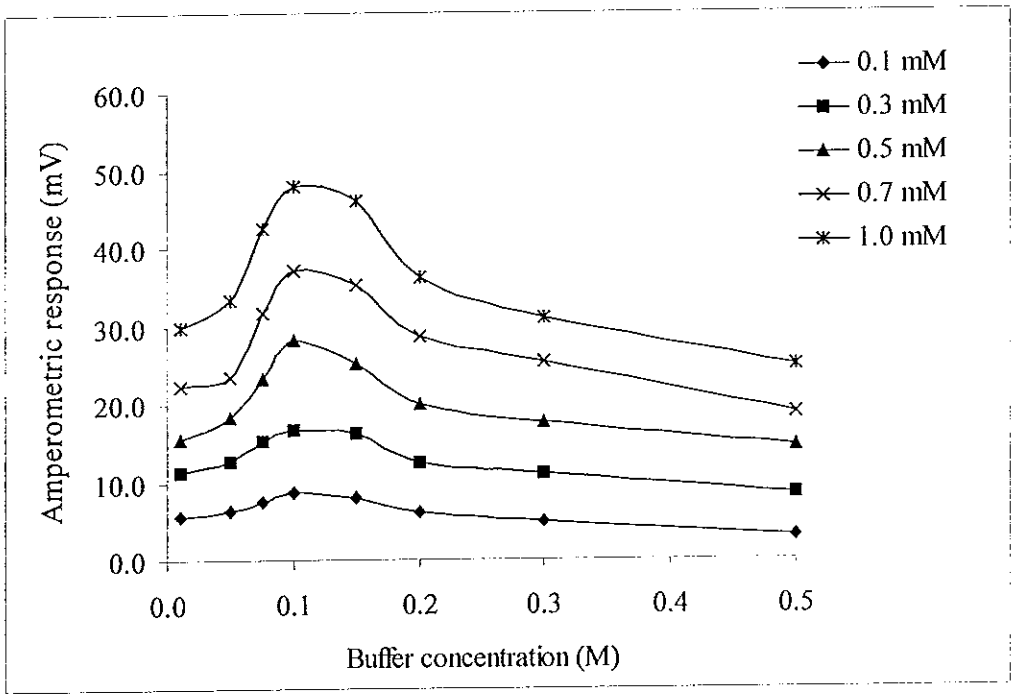


Figure 20 Responses of the glucose amperometric biosensor system using different buffer concentration

3.2.1.5 pH of buffer solution

The response of 0.1 to 1.0 mM standard glucose solution at different pH of phosphate buffer solution are shown in Table 9 and Figure 21. The maximum pH for this enzyme when immobilized on silica gel support is at pH 7.20. Therefore, phosphate buffer pH 7.20 was chosen for this work.

Table 9 Responses of the glucose amperometric biosensor system using different pH of buffer

Glucose concentration (mM)	Amperometric response (mV), different pH of buffer						
	6.00	6.50	7.00	**7.20**	7.40	7.60	8.00
	Mean±SD	Mean±SD	Mean±SD	**Mean±SD**	Mean±SD	Mean±SD	Mean±SD
0.1	6.3±0.2	6.7±0.3	7.9±0.1	**8.6±0.3**	7.3±0.2	5.0±0.0	4.6±0.3
0.3	12.5±0.4	13.5±0.3	14.9±0.1	**15.9±0.7**	14.1±0.3	12.2±0.1	9.0±0.2
0.5	18.4±0.1	19.3±0.4	22.5±0.5	**24.6±0.5**	21.0±0.2	17.6±0.1	15.0±0.1
0.7	23.7±0.3	24.6±0.7	29.4±0.5	**31.3±0.6**	27.5±0.5	23.7±0.1	20.9±0.5
1.0	30.5±0.5	32.5±0.5	37.6±0.5	**40.4±0.5**	35.6±0.5	27.4±0.5	26.9±0.6
Sensitivity (mV/mM)	27.0	28.5	33.4	**35.7**	31.6	25.9	25.6
R^2	0.9953	0.9971	0.9954	**0.9950**	0.9968	0.9961	0.9938
Analysis time(min)	9-11	9-11	9-11	**9-11**	9-11	9-11	9-11

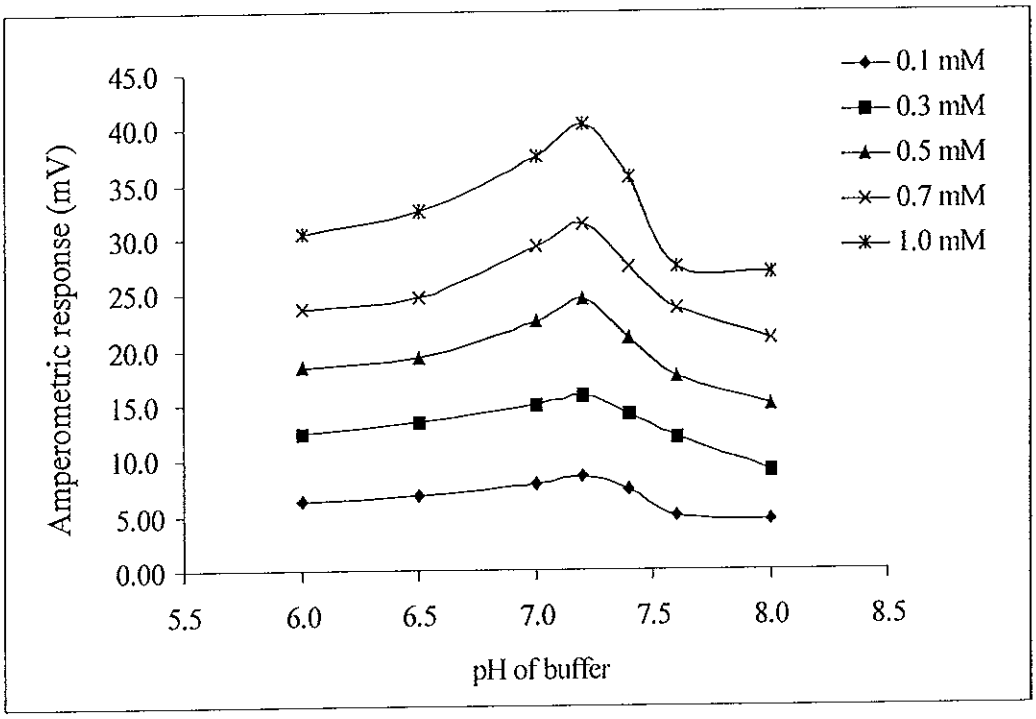


Figure 21 Responses of the glucose amperometric biosensor system using different pH of buffer

3.2.1.6 Effect of 0.9% (w/v) NaCl

The effect of 0.9% (w/v) NaCl on the amperometric system response was investigated in three situations. That is (I) no salt in any solution, (II) salt was added to standard glucose solution and (III) salt was added to standard glucose solution and buffer of sample line. The response of the enzyme reactor column to 0.1 to 1.0 mM standard glucose solution are shown in Table 10 and Figure 22. In all situations NaCl does not have significant effect on the responses of the amperometric system, and the sensitivity in all cases were not significant different (P=0.05). However, in case III the sensitivity was slightly better than the other two and it was chosen for further work. Furthermore, the added salt will also provide an isotonic condition for the plasma samples.

Table 10 Responses of the glucose amperometric biosensor system on the effect of 0.9%(w/v) NaCl, I) no salt in any solution, II) salt was added to standard glucose solution, III) salt was added to standard glucose solution and buffer of sample line

Glucose concentration (mM)	Amperometric response (mV), effect of 0.9%(w/v) NaCl at condition		
	I	II	**III**
	Mean±SD	Mean±SD	**Mean±SD**
0.1	6.9±0.1	9.0±0.3	**10.2±0.1**
0.3	13.9±0.4	16.9±0.1	**18.6±0.3**
0.5	20.6±0.4	23.9±0.4	**25.8±0.6**
0.7	28.0±0.7	31.0±0.2	**34.2±0.5**
1.0	35.7±0.5	39.7±0.5	**42.3±0.4**
Sensitivity (mV/mM)	32.343	34.098	**35.973**
R^2	0.9951	0.9962	**0.9925**
Analysis time (min)	9-11	9-11	**9-11**

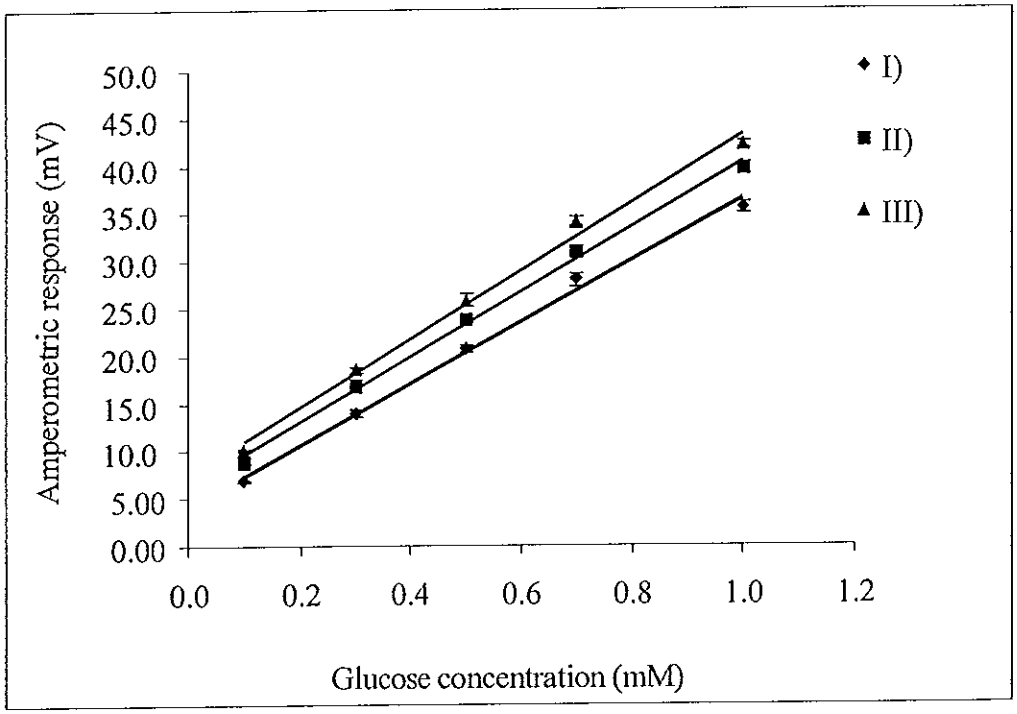


Figure22 Responses of the glucose amperometric biosensor system on the effect of 0.9%(w/v) NaCl

I) no salt in any solution

II) salt was added to standard glucose solution

III) salt was added to standard glucose solution and buffer of sample line

3.2.1.6.7 Sample Volume

The responses of 0.1 to 1 mM standard glucose solution at different sample volume *i.e.* 200, 300, 400, 500 and 600 µl pulsed into the system are shown in Table 11 and Figure 23 The response and the analysis time increased as the sample volume increased with the sensitivity highest at 600 µl. However, the analysis time was too long. Therefore, the sample volume of 500 µl was chosen because the sensitivity was only 3% lower than 600 µl but the analysis time was shorter.

Table 11 Responses of the glucose amperometric biosensor system using different sample volume

Glucose concentration (mM)	Amperometric response (mV), different sample volume				
	200 µl	300 µl	400 µl	**500 µl**	600 µl
	Mean ± SD	Mean ± SD	Mean ± SD	**Mean ± SD**	Mean ± SD
0.1	3.3±0.1	4.2±0.1	5.9±0.1	**8.0±0.0**	9.0 ±0.2
0.3	8.7±0.0	10.8±0.1	13.5±0.2	**17.0±0.0**	18.8 ±0.1
0.5	14.5±0.2	16.4±0.3	19.1±0.3	**23.6±0.1**	25.1±0.1
0.7	20.5±0.4	23.5±0.2	26.1±0.1	**31.5±0.2**	33.1±0.1
1.0	27.7±0.2	30.3±0.4	35.1±0.1	**40.2±0.2**	42.4 ±0.1
Sensitivity (mV/mM)	27.4	29.4	32.2	**35.7**	36.7
R^2	0.9985	0.9944	0.9982	**0.9939**	0.9931
Analysis time (min)	7-8	7-8	8-9	**9-11**	10-12

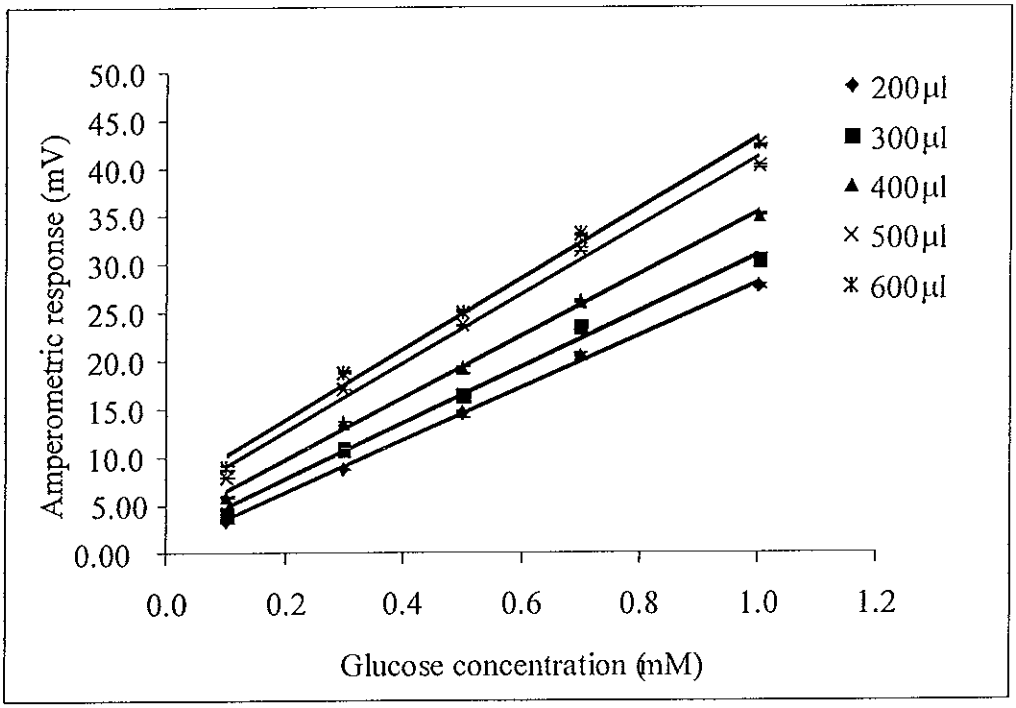


Figure 23 Responses of the glucose amperometric biosensor system using different sample volume

3.2.2 The flow injection system

3.2.2.1 Optimization of the flow rate

a) Flow rate of sample line

Table 12 and Figure 24show the amperometric response at different flow rate of the sample. The peak heights and sensitivity decreased as the flow rate increased. The slowest flow rate (0.2 ml min^{-1}) gave the highest sensitivity but also gave the longest analysis time. In this case the flow rate of 0.3 ml min^{-1} was chosen, because when compared to flow rate that gave the highest sensitivity the analysis time was shorter (9 min compared to 11 min) and the sensitivity was only 2% lower.

Table 12 Responses of the flow-injection amperometric biosensor system using different flow rate of the sample line with the flow rate of buffer line 0.75 ml min^{-1}

Glucose concentration (mM)	Amperometric response (mV), different flow rate of sample line				
	0.2 ml min^{-1}	**0.3 ml min^{-1}**	0.4 ml min^{-1}	0.5 ml min^{-1}	0.6 ml min^{-1}
	Mean ± SD	**Mean ± SD**	Mean ± SD	Mean ± SD	Mean ± SD
0.1	11.8±0.3	**10.0±0.2**	8.0±0.2	7.1±0.6	5.9±0.4
0.3	19.6±0.3	**18.3±0.5**	15.6±0.5	14.0±0.2	13.2±0.2
0.5	27.3±0.6	**24.6±0.5**	21.7±0.5	18.5±0.5	18.7±0.2
0.7	35.0±0.0	**31.7±0.2**	27.9±0.2	23.8±0.3	23.6±0.1
1.0	42.8±0.3	**41.0±0.2**	37.7±0.2	34.0±0.2	32.3±0.1
Sensitivity (mV/mM)	34.9	**34.2**	32.6	29.1	28.7
R^2	0.9922	**0.9973**	0.9986	0.9949	0.9958
Analysis time (min)	10-11	**8-9**	8-9	7-8	7-8

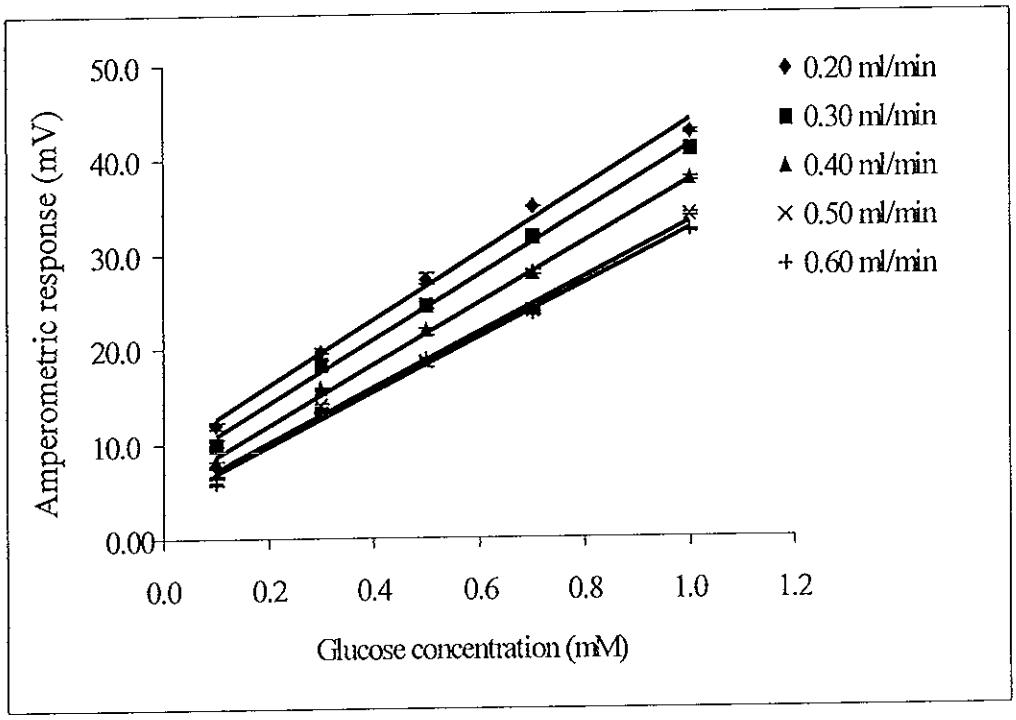


Figure 24 Responses of the flow-injection amperometric biosensor system using different flow rate of sample line

b) Flow rate of buffer line

The amperometric response of 0.1 to 1 mM standard glucose at different flow rate of buffer line are shown in Table 13 and Figure 25 The highest signals were obtained at 0.3 ml min^{-1}, The slowest flow rate. However, the analysis time was much longer than others. The flow rate of 0.75 ml min^{-1} was chosen because its only 18% lower than that of 0.3 ml min^{-1}, and the analysis time was much shorter (10 min compared to 25 min).

Table 13 Responses of the flow-injection amperometric biosensor system using different flow rate of the buffer line with flow rate of the sample line 0.40 ml min^{-1}

Glucose concentration (mM)	Amperometric response (mV), different flow rate of buffer line			
	0.30 ml/min	0.50 ml/min	**0.75 ml/min**	1.00 ml/min
	Mean ± SD	Mean ± SD	**Mean ± SD**	Mean ± SD
0.1	13.0±0.0	11.3±0.4	**9.5±0.2**	5.8±0.3
0.3	23.9±0.1	20.5±0.3	**17.8±0.1**	12.3±0.4
0.5	32.6±0.3	27.5±0.2	**23.2±0.1**	17.6±0.2
0.7	40.6±0.1	35.3±0.4	**31.1±0.1**	25.0±0.2
1.0	50.1±0.1	45.2±0.1	**39.9±0.1**	33.5±0.2
Sensitivity (mV/mM)	41.1	37.3	**33.5**	31.0
R^2	0.9907	0.9962	**0.9956**	0.9983
Analysis time (min)	20-25	13-18	**8-10**	7-8

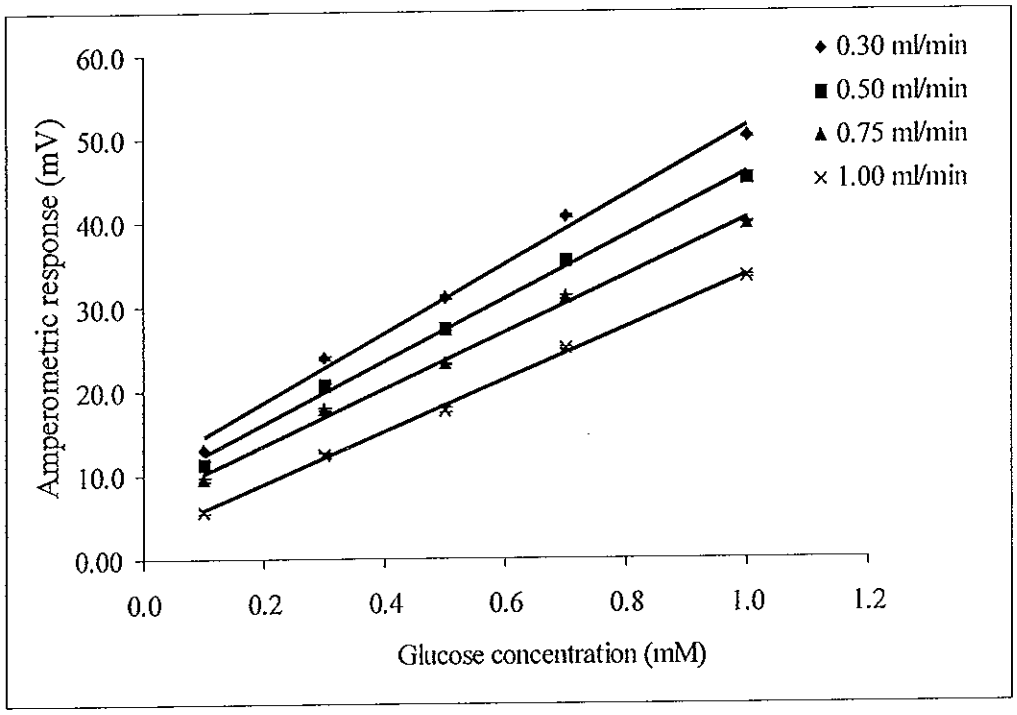


Figure 25 Responses of the flow-injection amperometric biosensor system using different flow rate of buffer line

3.2.2.2 Sample volume

The effect of the sample volume, 200 to 500 µl, of standard glucose solution are shown in Table 14 and Figure 26 The response increased as the sample volume increased. Therefore, a 500 µl of sample volume was chosen because the sensitivity was much higher than others while the analysis time was not much different.

Table 14 Responses of the flow-injection amperometric biosensor system using different sample volume

Glucose concentration (mM)	Amperometric response (mV), different sample volume			
	200 µl	300 µl	400 µl	**500 µl**
	Mean ± SD	Mean ± SD	Mean ± SD	**Mean ± SD**
0.1	3.8±0.2	4.8±0.2	7.1±0.1	**9.0±0.0**
0.3	9.3±0.3	11.5±0.1	14.6±0.1	**17.3±0.1**
0.5	15.3±0.1	15.8±0.1	20.2±0.2	**23.2±0.1**
0.7	21.7±0.2	23.4±0.4	27.0±0.0	**30.0±0.1**
1.0	28.2±0.3	31.9±0.1	34.6±0.1	**38.5±0.1**
Sensitivity (mV/mM)	27.7	30.1	30.5	**32.5**
R^2	0.9946	0.9957	0.9951	**0.9952**
Analysis time (min)	7-8	8-9	8-10	**8-10**

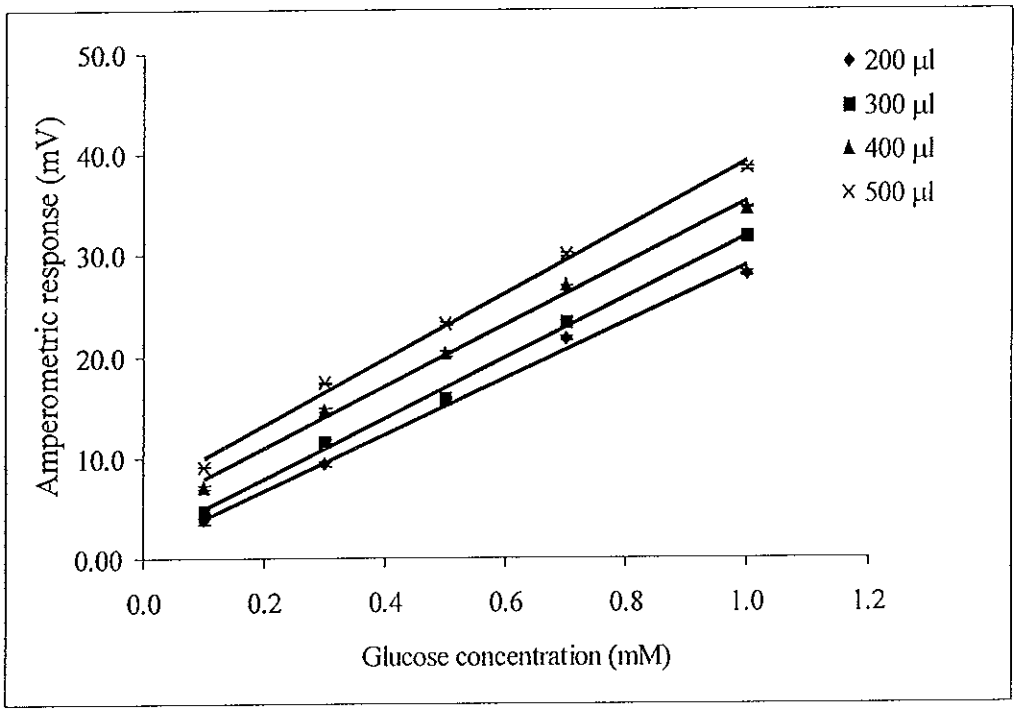


Figure 26 Responses of the flow-injection amperometric biosensor system using different sample volume

3.2.3 Comparison of the flow-through and flow-injection systems

The same glucose standard solutions (0.1 to 1.0 mM) were tested by the two proposed systems at their optimum conditions, *i.e.* 0.3 and 0.75 ml min^{-1} flow rate of sample and buffer lines respectively and 500 µl sample volume. The results are shown in Table 15 and Figure 27. Sensitivity, precision, limit of detection and analysis time were considered. Both systems provided the same detection limit of 0.1 mM and they also had similar analysis time between 8-10 min. However, the sensitivity of the flow injection system was slightly higher than the flow-through system (7%) and the linearity was better (R^2 =0.9955). This may be because in the flow-through system the sample was introduced into the system as a pulse by timing the passing of the sample for 100 sec, *i.e.* 500 µl. The tube was moved by hand from the buffer to the sample and back. Therefore, the volume of sample may not be as accurate as the flow injection system. This is showed in the higher %RSD than the flow-injection system. Therefore, the flow-injection system was used for further investigation.

Table 15 Comparison of the response of the flow-through and flow-injection amperometric biosensor system

Glucose concentration (mM)	Amperometric response (mV)	
	Flow-through system	Flow-injection system
	Mean±SD	**Mean±SD**
0.1	7.3±0.4	**8.3±0.2**
0.3	15.2±0.2	**16.2±0.1**
0.5	20.0±0.2	**23.2±0.2**
0.7	28.2±0.4	**29.9±0.1**
1.0	36.2±0.4	**38.6±0.1**
Sensitivity (mV/mM)	32.07	**33.64**
R^2	0.9937	**0.9958**
Max. %RSD	5.45	**2.42**
Analysis time (min)	8-10	**8-10**

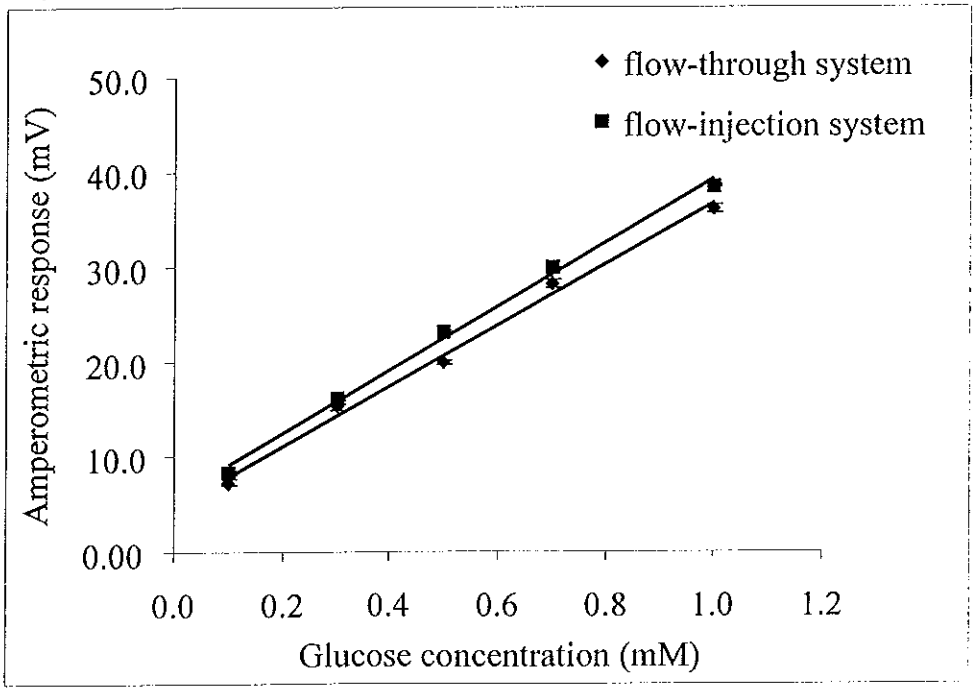


Figure 27 Comparison of the response of the flow-through and flow-injection amperometric biosensor system

3.2.4 Stability of the enzyme reactor column

When immobilized enzyme was used for a long period of time its response may change (Cabral and Kennedy, 1991). The long term stability of immobilized glucose oxidase was tested intermittently over a 5 month period (312 operation time) by monitor its response to 500 µl of standard glucose solutions. The responses are shown in Table 16 and Figure 28. Although the sensitivity of the reactor column gradually decreased as the operation time increased it was still at a satisfactory level (67% of the original sensitivity) after such a long period of operation time.

Table 16 Responses of the amperometric biosensor system at different operation time of the enzyme reactor

Glucose concentration (mM)	Amperometric response (mV), operation time (h)					
	0	85	120	194	262	312
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
0.1	8.6±0.3	7.7±0.3	7.2±0.3	6.6±0.1	6.3±0.3	5.8±0.3
0.3	15.9±0.6	14.3±0.3	12.2±0.3	10.8±0.3	9.8±0.3	9.0±0.5
0.5	24.5±0.5	22.0±0.5	20.2±0.3	18.3±0.6	16.8±0.3	15.3±0.3
0.7	31.3±0.5	28.8±0.3	25.5±0.5	23.5±0.5	22.0±0.5	20.5±0.5
1.0	40.4±0.5	37.5±0.5	33.7±0.3	30.7±0.6	28.8±0.3	26.5±0.5
Sensitivity (mV/mM)	35.74	33.64	30.04	25.53	25.90	23.92
R^2	0.9950	0.9974	0.9947	0.9929	0.9923	0.9912
Analysis time (min)	8-9	8-9	8-9	8-9	8-9	8-9

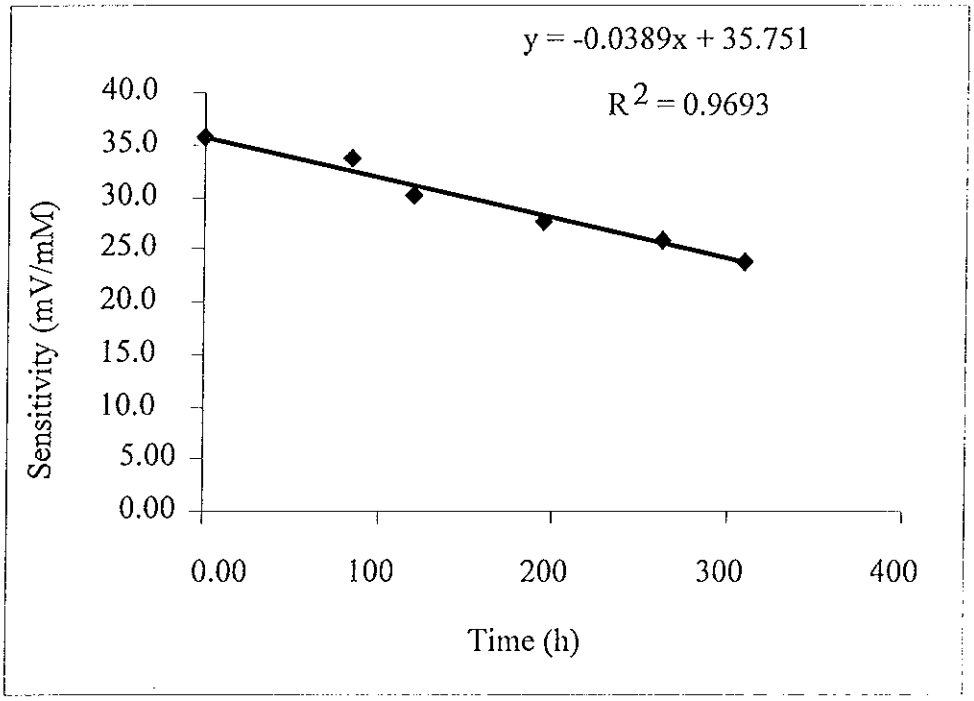


Figure 28 Sensitivity of the amperometric biosensor system at different operation time of the enzyme reactor

3.2.5 Reproducibility of immobilized enzyme

Table 17 and Figure 29 show the responses of two columns of glucose oxidase immobilized in the same batch. Table 18 and Figure 30 show the responses of the glucose oxidase immobilized in two different batches. The responses of these columns were not differed significantly (P=0.05). That is, this immobilization technique, when carefully monitor provides a good reproducibility.

Table 17 Responses of different columns using immobilized enzyme from same batch

Glucose concentration (mM)	Amperometric response (mV)	
	First column	**Second column**
	Mean±SD	Mean±SD
0.1	8.6±0.3	8.3±0.3
0.3	15.9±0.6	16.2±0.1
0.5	24.5±0.5	23.3±0.3
0.7	31.3±0.6	29.9±0.1
1.0	40.4±0.5	38.5±0.1
Sensitivity (mV/mM)	35.74	33.64
R^2	0.9950	0.9958
Analysis time (min)	9-11	8-10

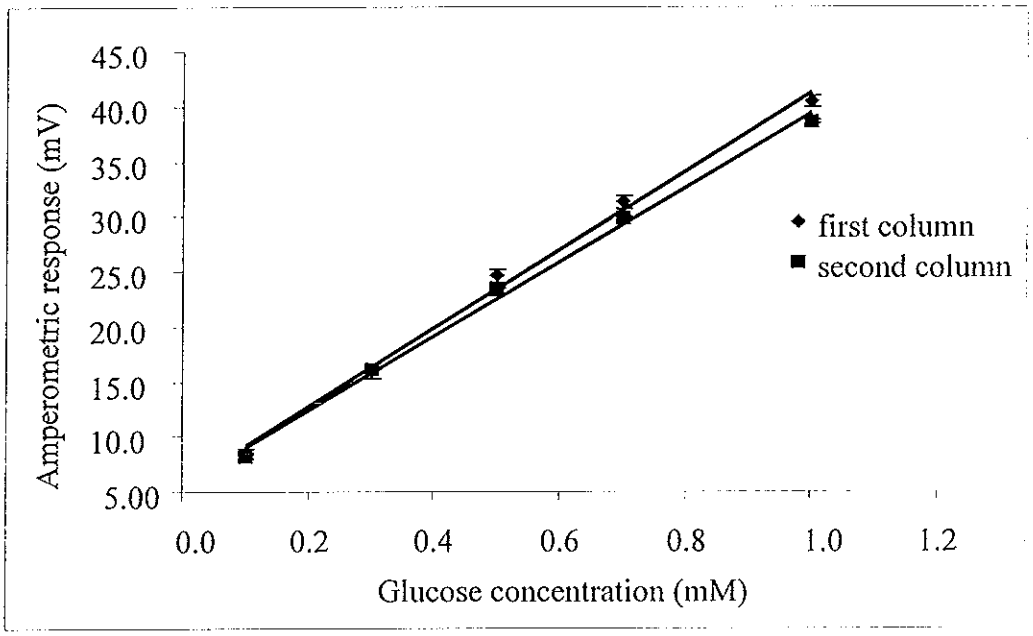


Figure 29 Responses from different columns using immobilized enzyme from same batch

Table 18 Responses of different columns using immobilized enzyme from different batches

Glucose concentration (mM)	Amperometric response (mV)	
	First batch	**Second batch**
	Mean±SD	Mean±SD
0.1	8.3±0.3	8.3±0.3
0.3	16.2±0.1	16.1±0.1
0.5	23.3±0.3	24.0±0.0
0.7	29.9±0.1	31.9±0.1
1.0	38.5±0.1	40.0±0.0
Sensitivity (mV/mM)	33.64	35.78
R^2	0.9958	0.9927
Analysis time (min)	8-10	8-10

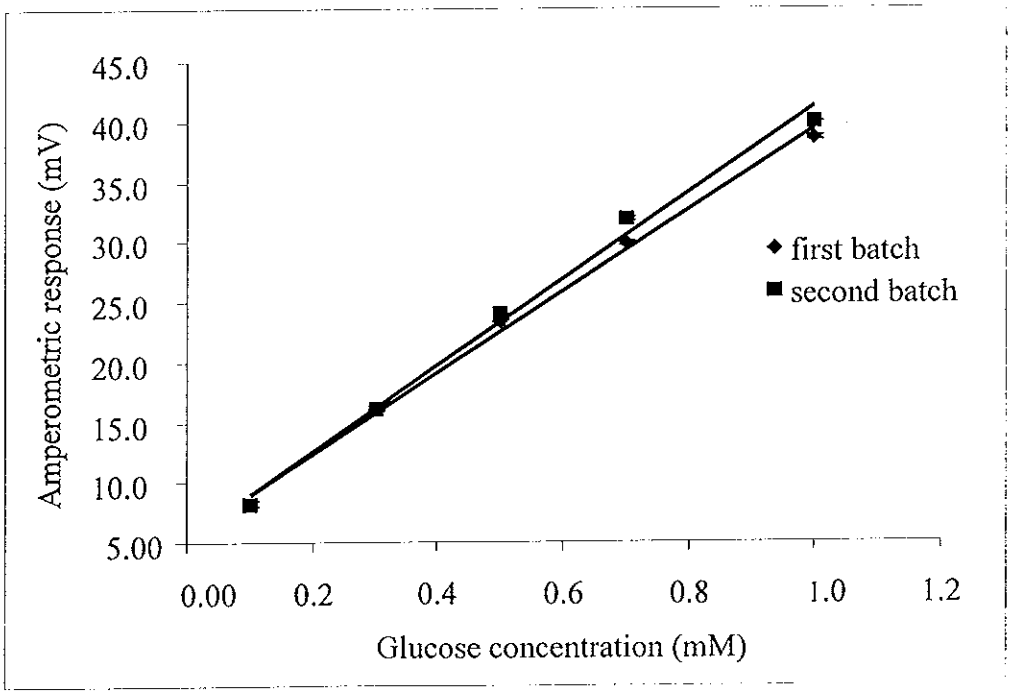


Figure 30 Responses different columns using immobilized enzyme from the different batch

3.2.6 Linearity of the responses of glucose oxidase

The normal glucose concentration in human plasma is between 4.4 to 6.2 mM (Atanasov and Wilkins, 1994) while for those with diabetes is much higher, up to 11 mM (Mayne, 1994). From other experiments done in this laboratory (Saelim, 2000; Limbut, 2001) the sample needed to be diluted at least 10 times to reduce its viscosity. Therefore, the detection limit of the system needs to be at least 0.4 mM and a wide linear range is prefered.

The linearity of the response of the system to different concentration of standard glucose solution are shown in Table 19 and Figure 31. The linear range was 0.1-6 mM. So, the system can be used for blood glucose analysis.

Table 19 Response of the system to different concentration of glucose

Concentraion of glucose (mM)	Response (mM) Mean ± SD
0.1	8.3±0.3
0.3	16.1±0.1
0.5	24.0±0.0
0.7	31.9±0.1
1.0	40.0±0.0
2.0	71.7±0.3
3.0	105.±0.3
4.0	139±0.4
5.0	165±0.6
6.0	202±0.3
6.5	210±0.1
7.0	223±0.2
8.0	232±0.0
9.0	240±0.3
Sensitivity (mV/mM) of 0.1-6.0 mM glucose standard solution	32.44
R^2	0.9993
Analysis time (min)	8-11

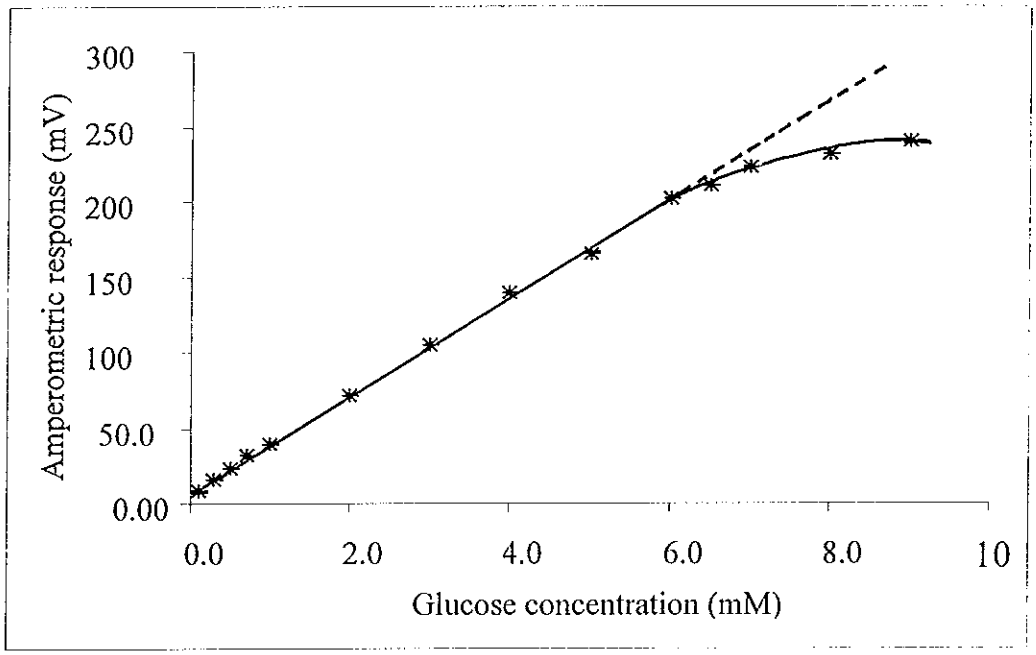


Figure 31 Response of the system to different concentration of glucose

3.3 Calorimetric system

3.3.1 Flow through system

3.3.1.1 Optimization of the flow rate

a) Flow rate without a dialyser

Table 20 and Figure 32 show the effect of the flow rate without dialyser on the calorimetric system for the two enzyme reactor columns with immobilized glucose oxidase and glucose oxidase co-immobilized with catalase. The system with two enzymes gave higher responses than when only one enzyme was used. For both reactor columns, the peak heights and the sensitivities increases as the flow rate decreased. The slowest flow rate (0.25 ml min^{-1}) gave the highest sensitivity but also gave the longest analysis time (25-30 min). In this case the flow rate of 0.75 ml min^{-1} was chosen. Although its sensitivity was lower than at 0.25 ml min^{-1} it was still quite high and its analysis time was much shorter.

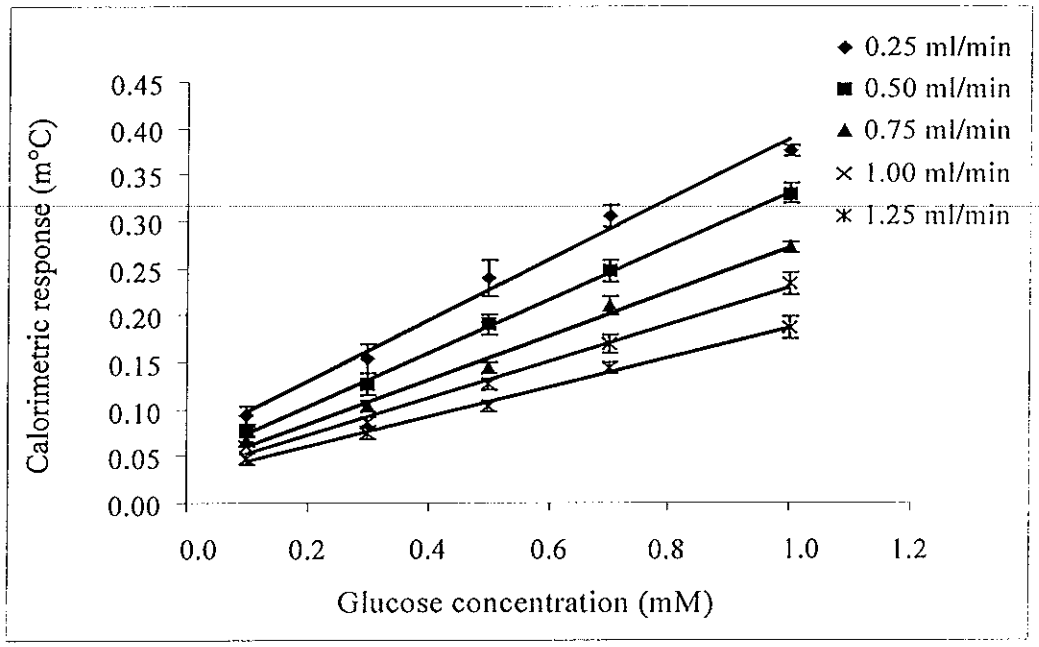
Table 20 Responses of the glucose calorimetric biosensor system using different flow rate without a dialyser

(a) immobilized glucose oxidase

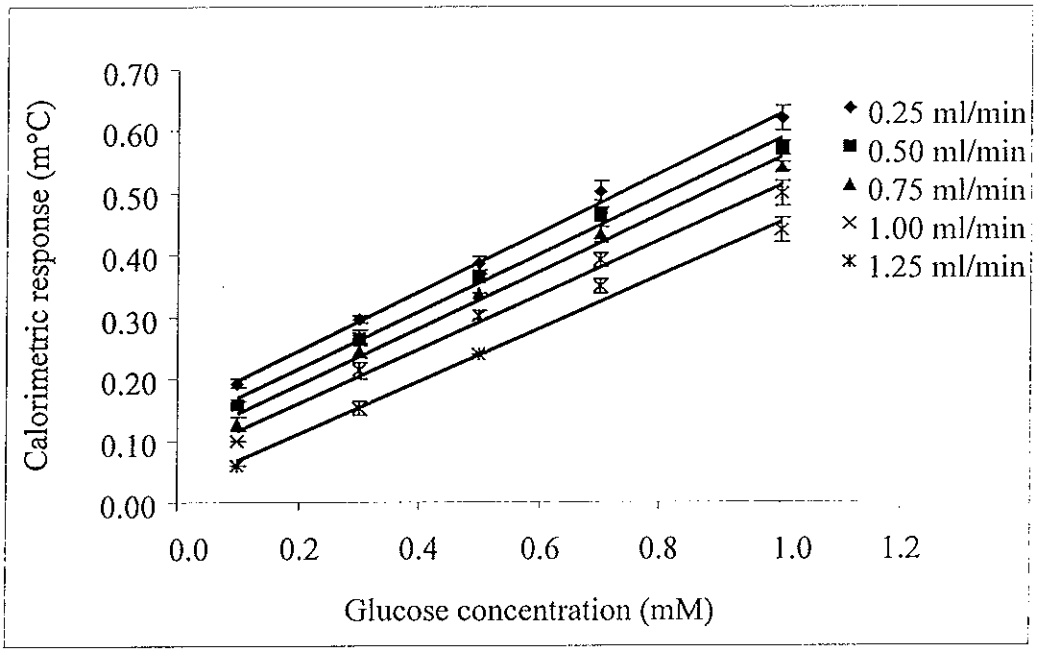
Glucose concentration (mM)	Calorimetric response (m°C), different flow rate without dialyser				
	0.25 ml/min	0.50 ml/min	**0.75 ml/min**	1.00 ml/min	1.25 ml/min
	Mean ± SD	Mean ± SD	**Mean ± SD**	Mean ± SD	Mean ± SD
0.1	0.09±0.01	0.08±0.01	**0.07±0.01**	0.06±0.00	0.05±0.01
0.3	0.15±0.02	0.13±0.01	**0.10±0.01**	0.09±0.01	0.07±0.01
0.5	0.24±0.02	0.19±0.01	**0.14±0.01**	0.13±0.01	0.10±0.01
0.7	0.31±0.01	0.25±0.01	**0.21±0.01**	0.17±0.01	0.14±0.01
1.0	0.38±0.01	0.33±0.01	**0.27±0.01**	0.23±0.01	0.19±0.01
Sensitivity (m°C/mM)	0.3243	0.2847	**0.2365**	0.1963	0.1590
R^2	0.9878	0.9992	**0.9908**	0.9938	0.9962
Analysis time (min)	25-30	17-22	**8-9**	7-8	7-8

(b) glucose oxidase co-immobilized with catalase

Glucose concentration (mM)	Calorimetric response (m°C), different flow rate without dialyser				
	0.25 ml/min	0.50 ml/min	**0.75 ml/min**	1.00 ml/min	1.25 ml/min
	Mean ± SD	Mean ± SD	**Mean ± SD**	Mean ± SD	Mean ± SD
0.1	0.19±0.01	0.16±0.01	**0.13±0.01**	0.10±0.00	0.06±0.00
0.3	0.30±0.01	0.26±0.01	**0.25±0.01**	0.21±0.01	0.15±0.01
0.5	0.39±0.01	0.37±±0.01	**0.34±0.00**	0.30±0.01	0.24±0.00
0.7	0.50±0.02	0.47±0.01	**0.43±0.01**	0.39±0.01	0.35±0.01
1.0	0.62±0.02	0.57±0.01	**0.54±0.01**	0.50±0.02	0.44±0.02
Sensitivity (m°C/mM)	0.4795	0.4661	**0.4601**	0.4422	0.4313
R^2	0.9961	0.9926	**0.9906**	0.9919	0.9906
Analysis time (min)	25-30	17-22	**8-9**	7-8	7-8



(a)



(b)

Figure 32 Responses of the glucose calorimetric biosensor system using different flow rate without dialyser (a) enzyme reactor column with immobilized glucose oxidase (b) enzyme reactor column with glucose oxidase co-immobilized catalase

b) Flow rate of the sample line with a dialyser

The responses at different flow rates for the two enzyme reactor columns are shown in Table 21 and Figure 33. For both reactors, the highest signals were obtained at the slowest flow rate 0.20 ml min^{-1}. However, the analysis time was much longer than others. At 0.40 ml min^{-1} the sensitivity was only lower than 0.2 ml min^{-1} by 14% and 11% for the reactor columns with glucose oxidase and with glucose oxidase co-immobilized with catalase respectively but the analysis time was shorter by 3-4 min. So, it was chosen to be used in further analysis.

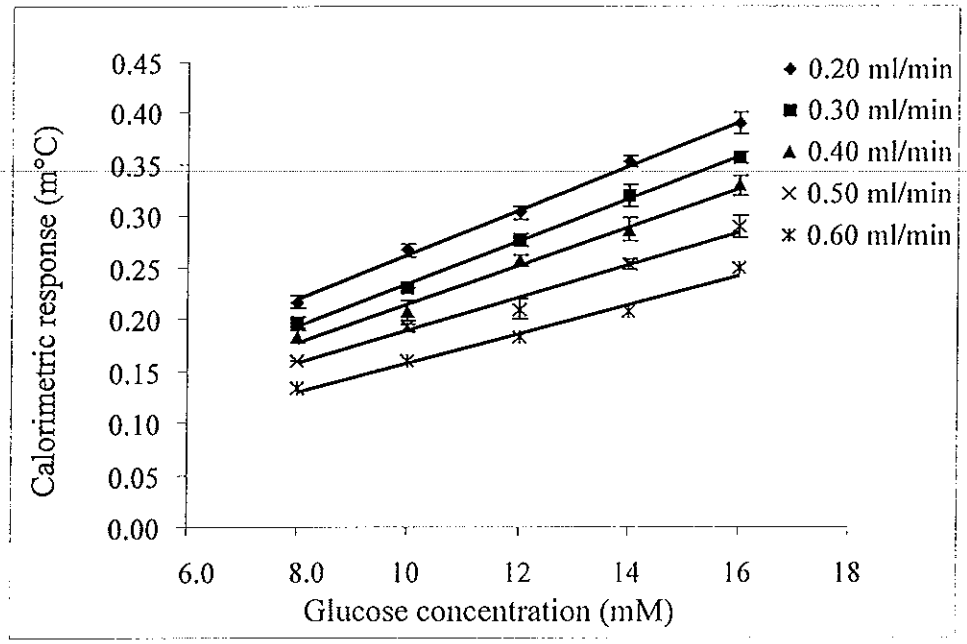
Table 21 Responses of the glucose calorimetric biosensor system using different flow rate of the sample line with buffer line flow rate 0.75 ml min^{-1}

(a) immobilized glucose oxidase

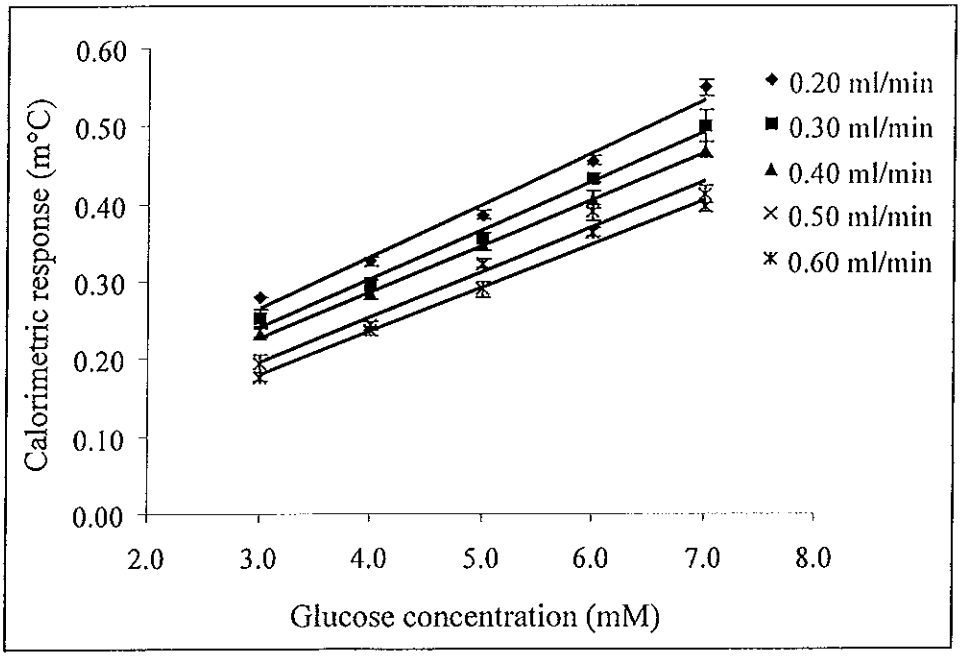
Glucose concentration (mM)	Amperometric response (m°C), different flow rate of sample line				
	0.20 ml/min	0.30 ml/min	**0.40 ml/min**	0.50 ml/min	0.60 ml/min
	Mean ± SD	Mean ± SD	**Mean ± SD**	Mean ± SD	Mean ± SD
8.0	0.22±0.01	0.20±0.01	**0.18±0.01**	0.16±0.00	0.13±0.01
10	0.27±0.01	0.23±0.00	**0.21±0.01**	0.19±0.01	0.16±0.01
12	0.30±0.01	0.28±0.01	**0.26±0.01**	0.21±0.01	0.18±0.01
14	0.35±0.01	0.32±0.01	**0.29±0.01**	0.25±0.01	0.21±0.01
16	0.39±0.01	0.36±0.01	**0.33±0.01**	0.29±0.01	0.25±0.01
Sensitivity (m°C/mM)	0.0217	0.0205	**0.0187**	0.0160	0.0058
R^2	0.9972	0.9974	**0.9902**	0.9834	0.9828
Analysis time (min)	11-13	9-11	**8-9**	8-9	8-9

(b) glucose oxidase co-immobilized with catalase

Glucose concentration (mM)	Amperometric response (m°C), different flow rate of sample line				
	0.20 ml/min	0.30 ml/min	**0.40 ml/min**	0.50 ml/min	0.60 ml/min
	Mean ± SD	Mean ± SD	**Mean ± SD**	Mean ± SD	Mean ± SD
3.0	0.28±0.00	0.25±0.01	**0.23±0.01**	0.19±0.01	0.18±0.01
4.0	0.33±0.01	0.30±0.01	**0.28±0.01**	0.24±0.01	0.24±0.01
5.0	0.39±0.01	0.36±0.01	**0.35±0.01**	0.32±0.01	0.29±0.01
6.0	0.46±0.01	0.43±0.01	**0.41±0.01**	0.39±0.01	0.36±0.01
7.0	0.55±0.01	0.50±0.02	**0.47±0.01**	0.41±0.01	0.40±0.01
Sensitivity (m°C/mM)	0.0670	0.0630	**0.0597**	0.0587	0.0567
R^2	0.9830	0.9908	**0.9984**	0.9757	0.9908
Analysis time (min)	11-13	9-11	**8-9**	8-9	8-9



(a)



(b)

Figure 33 Responses of the glucose calorimetric biosensor system using different flow rate of sample line (a) enzyme reactor column with immobilized glucose oxidase (b) enzyme reactor column with glucose oxidase co-immobilized catalase

c) Flow rate of the buffer line with a dialyser

The responses of the two enzyme reactor columns, to standard glucose solution at different flow rates of the buffer line are shown in Table 22 and Figure 34. For both reactor columns, the peak heights and the sensitivity decrease as the flow rate increased. The flow rate of 0.75 ml min^{-1} was chosen because when compared to the flow rate that gave the highest sensitivity (0.40 ml min^{-1}) the analysis time was much shorter, 8-9 min c.f. 24-29 min. Also the sensitivity was only slightly lower, 4% for the reactor column with immobilized glucose oxidase and 12% lower for the reactor column with glucose oxidase co-immobilized catalase.

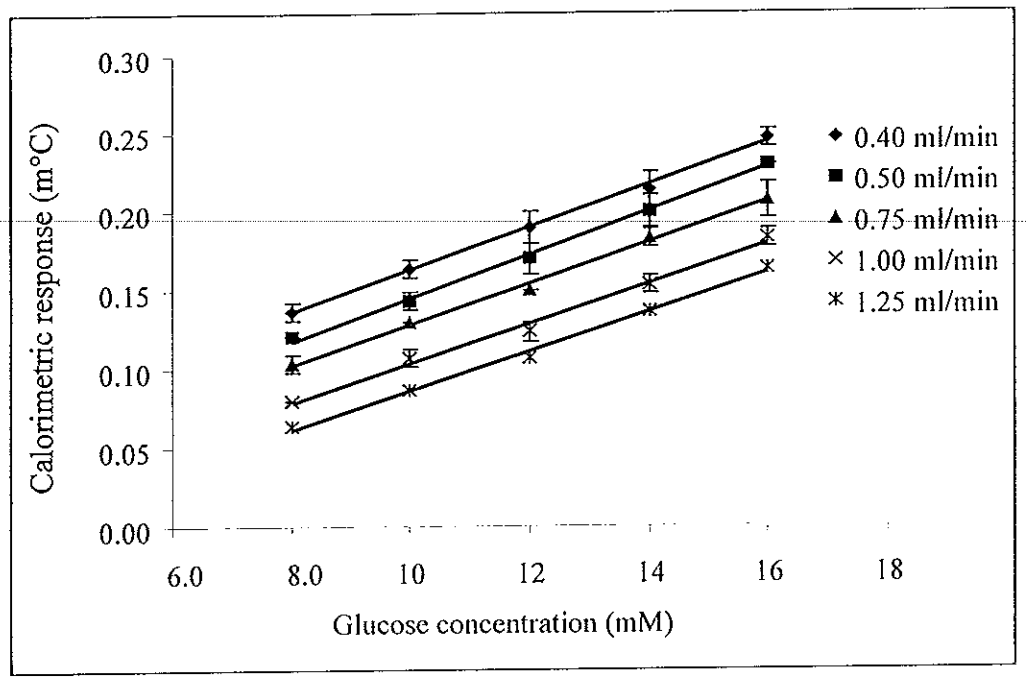
Table 22 Responses of the glucose calorimetric biosensor system using different flow rate of buffer line with buffer line flow rate 0.40 ml min^{-1}

(a) immobilized glucose oxidase

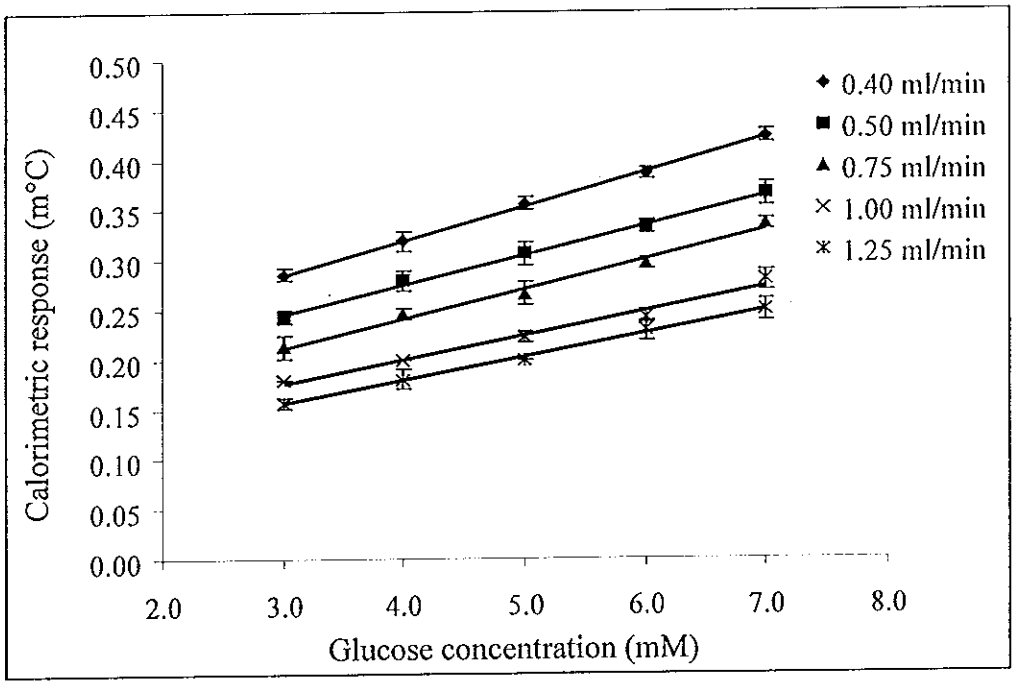
Glucose concentration (mM)	Amperometric response (m°C), different flow rate of buffer line				
	0.40 ml/min	0.50 ml/min	**0.75 ml/min**	1.00 ml/min	1.25 ml/min
	Mean ± SD	Mean ± SD	**Mean ± SD**	Mean ± SD	Mean ± SD
8.0	0.14±0.01	0.12±0.00	**0.10±0.01**	0.08±0.00	0.06±0.01
10	0.16±0.01	0.14±0.01	**0.13±0.00**	0.11±0.01	0.09±0.01
12	0.19±0.01	0.17±0.01	**0.15±0.00**	0.12±0.01	0.11±0.01
14	0.21±0.01	0.20±0.01	**0.18±0.01**	0.15±0.01	0.14±0.01
16	0.25±0.01	0.23±0.00	**0.21±0.01**	0.18±0.01	0.16±0.01
Sensitivity (m°C/mM)	0.0135	0.0138	**0.0130**	0.0127	0.0125
R^2	0.9971	0.9972	**0.9954**	0.9911	0.9952
Analysis time (min)	24-29	18-24	**8-9**	8-9	8-9

(b) glucose oxidase co-immobilized with catalase

Glucose concentration (mM)	Amperometric response (m°C), different flow rate of buffer line				
	0.40 ml/min	0.50 ml/min	**0.75 ml/min**	1.00 ml/min	1.25 ml/min
	Mean ± SD	Mean ± SD	**Mean ± SD**	Mean ± SD	Mean ± SD
3.0	0.29±0.01	0.24±0.01	**0.21±0.01**	0.18±0.00	0.16±0.01
4.0	0.32±0.01	0.28±0.01	**0.25±0.01**	0.20±0.00	0.18±0.01
5.0	0.36±0.01	0.31±0.01	**0.27±0.01**	0.22±0.01	0.20±0.00
6.0	0.39±0.01	0.33±0.01	**0.30±0.01**	0.24±0.01	0.23±0.01
7.0	0.42±0.01	0.37±0.01	**0.34±0.01**	0.28±0.01	0.25±0.01
Sensitivity (m° C/mM)	0.0340	0.0300	**0.0297**	0.0243	0.0237
R^2	0.9992	0.9966	**0.9886**	0.9847	0.9962
Analysis time (min)	24-29	18-24	**8-9**	8-9	8-9



(a)



(b)

Figure 34 Responses of the glucose calorimetric biosensor system using different flow rate of buffer line (a) enzyme reactor column with immobilized glucose oxidase (b) enzyme reactor column with glucose oxidase co-immobilized catalase

3.3.1.2 Type of buffer solution

The effect of type of buffer solution on the calorimetric system response was investigated by using four buffer solutions. The responses of standard glucose solution in the different types of buffer solution for both enzyme reactor columns are shown in Table 23 and Figure 35. In both reactor column, the peak height and the sensitivities when using phosphate buffer were higher than those of K_2HPO_4-NaOH, Tris-HCl and EDTA buffers. This is because not all of the heat generated during the oxidation of glucose is detected by the thermistor. Part of this heat will be absorbed in the deprotonation reaction of buffer. Since the endothermic deprotonation reaction required 47.45 kJ mol^{-1} for Tris-HCl and 3.43 kJ mol^{-1} for phosphate buffer (Edsall and Gutfreund, 1983; Bjarnason *et al.*,1998) more heat would be absorbed by Tris-HCl. Therefore, a smaller response would be detected. For EDTA and K_2HPO_4-NaOH buffers there is no data, but it is expected the heat required for endothermic deprotonation reaction would be more than phosphate. So, phosphate was chosen for this analysis.

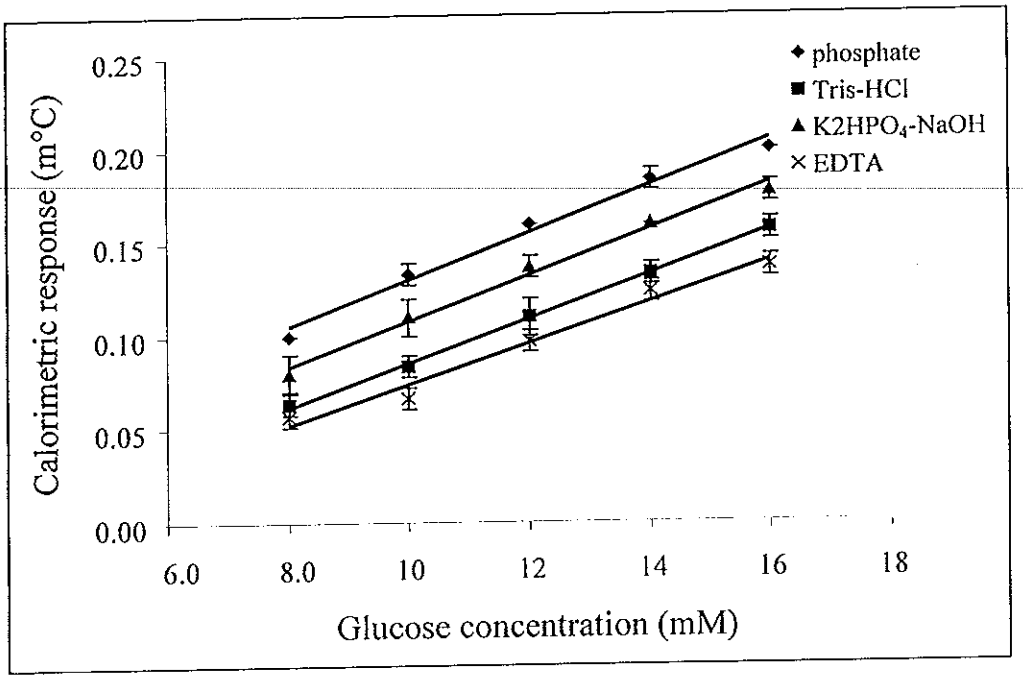
Table 23 Responses of the glucose calorimetric biosensor system using different type of buffer solution

(a) immobilized glucose oxidase

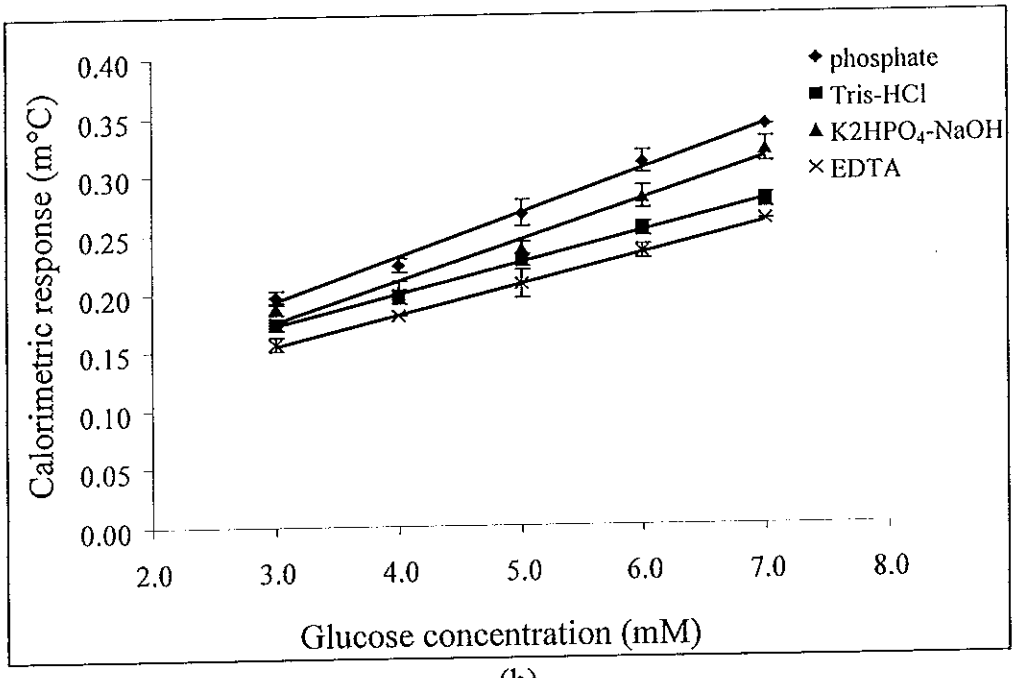
Glucose concentration (mM)	Amperometric response (m°C), different type of buffer solution			
	phosphate	Tris-HCl	K_2HPO_4-NaOH	EDTA
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
8.0	**0.10±0.00**	0.0.6±0.01	0.08±0.01	0.06±0.01
10	**0.13±0.01**	0.08±0.01	0.11±0.01	0.07±0.01
12	**0.16±0.00**	0.11±0.01	0.14±0.01	0.10±0.01
14	**0.18±0.01**	0.13±0.01	0.16±0.00	0.12±0.01
16	**0.20±0.00**	0.16±0.01	0.18±0.01	0.14±0.01
Sensitivity (m°C/mM)	**0.0125**	0.0118	0.0122	0.0108
R^2	**0.9848**	0.9986	0.9890	0.9762
Analysis time (min)	**8-9**	8-9	8-9	8-9

(b) glucose oxidase co-immobilized with catalase

Glucose concentration (mM)	Amperometric response (m°C), different typeof buffer solution			
	phosphate	Tris-HCl	K_2HPO_4-NaOH	EDTA
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
3.0	**0.20±0.01**	0.17±0.01	0.19±0.01	0.16±0.01
4.0	**0.22±0.01**	0.20±0.01	0.20±0.01	0.18±0.00
5.0	**0.27±0.01**	0.23±0.01	0.24±0.01	0.21±0.01
6.0	**0.31±0.01**	0.25±0.01	0.28±0.01	0.23±0.01
7.0	**0.34±0.00**	0.28±0.01	0.32±0.01	0.26±0.00
Sensitivity (m°C/mM)	**0.0.373**	0.0263	0.0347	0.026
R^2	**0.9939**	0.9982	0.9734	0.9993
Analysis time (min)	**8-9**	8-9	8-9	8-9



(a)



(b)

Figure 35 Responses of the glucose calorimetric biosensor system using different type of buffer solution (a) enzyme reactor column with immobilized glucose oxidase (b) enzyme reactor column with glucose oxidase co-immobilized catalase

3.3.1.3 Buffer concentration

The responses of standard glucose solution using phosphate buffer pH 7.00 between 0.01 to 0.20 M are shown in Table 24 and Figure 36. In both reactor columns the peak height and the sensitivity increased as the buffer concentration increased from 0.01 to 0.10 M and then decreased at higher buffer concentration. It is possible that at high buffer concentration, *i.e.* high viscosity fluid, the permeation rate through the dialysis membrane is low resulting in a lower response. On the other hand for low buffer concentration the buffer capacity is too low and its pH would change during the measurement and this would have same effect on the response of the enzyme. The buffer concentration of 0.10 M phosphate pH 7.00 was chosen for further work.

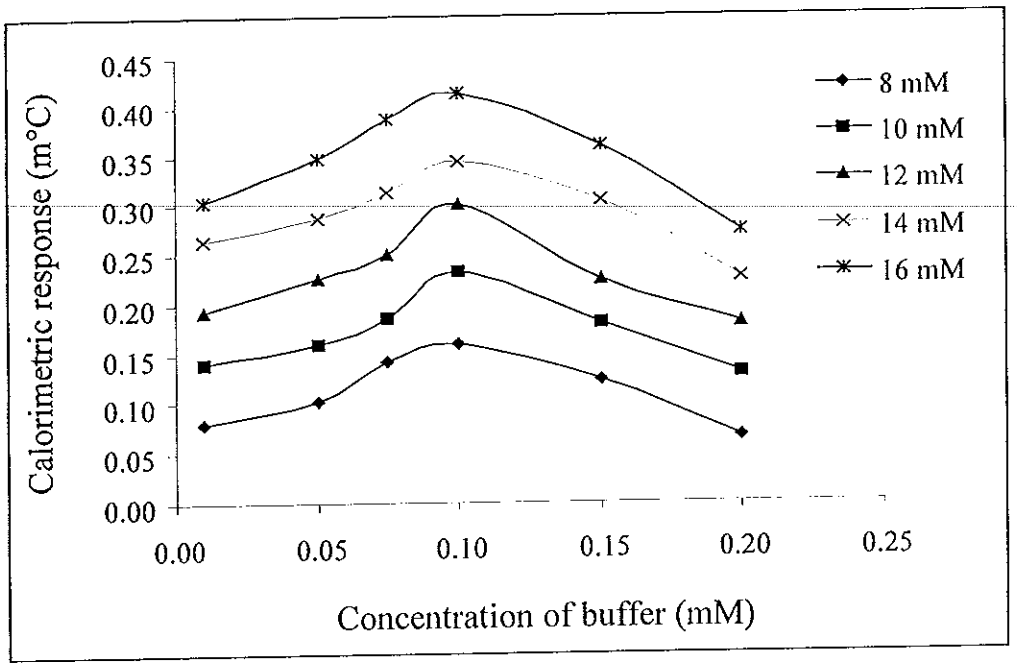
Table 24 Responses of the glucose calorimetric biosensor system using different buffer concentration

(a) immobilized glucose oxidase

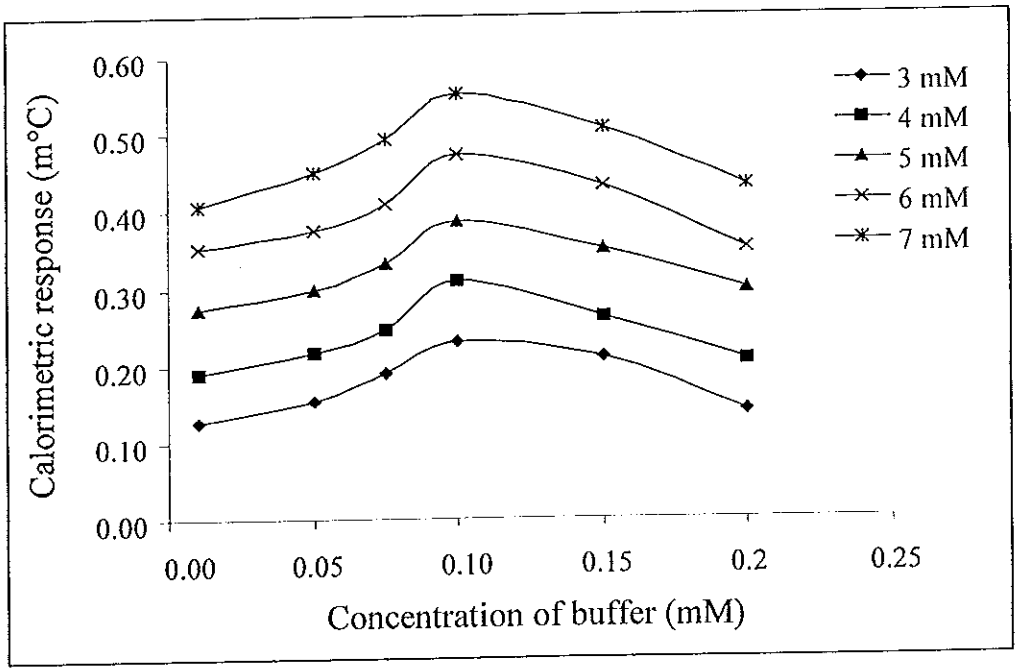
Glucose concentration (mM)	Amperometric response (m°C), different buffer concentration					
	0.01 M	0.05 M	0.075 M	**0.10 M**	0.15 M	0.20 M
	Mean ± SD	Mean ± SD	Mean ± SD	**Mean ± SD**	Mean ± SD	Mean ± SD
8.0	0.08±0.01	0.10±0.01	0.14±0.01	**0.16±0.00**	0.12±0.01	0.07±0.01
10	0.14±0.01	0.16±0.01	0.19±0.0	**0.23±0.01**	0.18±0.01	0.13±0.01
12	0.19±0.01	0.23±0.01	0.25±0.01	**0.30±0.01**	0.22±0.01	0.18±0.01
14	0.26±0.01	0.29±0.01	0.31±0.01	**0.34±0.01**	0.30±0.01	0.23±0.01
16	0.30±0.01	0.35±0.01	0.39±0.01	**0.41±0.01**	0.36±0.02	0.27±0.01
Sensitivity (m°C/mM))	0.0285	0.0307	0.0307	**0.0308**	0.0298	0.0255
R^2	0.9950	0.9995	0.9926	**0.9938**	0.9922	0.9956
Analysis time (min)	8-9	8-9	8-9	**8-9**	8-10	8-10

(b) glucose oxidase co-immobilized with catalase

Glucose concentration (mM)	Amperometric response (m°C), different buffer concentration					
	0.01 M	0.05 M	0.075 M	**0.10 M**	0.15 M	0.20 M
	Mean ± SD	Mean ± SD	Mean ± SD	**Mean ± SD**	Mean ± SD	Mean ± SD
3.0	0.13±0.01	0.15±0.01	0.19±0.01	**0.23±0.01**	0.21±0.01	0.14±0.00
4.0	0.19±0.01	0.22±0.01	0.25±0.01	**0.31±0.01**	0.26±0.02	0.21±0.01
5.0	0.27±0.01	0.30±0.01	0.33±0.01	**0.39±0.01**	0.35±0.01	0.30±0.02
6.0	0.35±0.01	0.38±0.01	0.41±0.01	**0.47±0.01**	0.43±0.01	0.35±0.01
7.0	0.41±0.01	0.45±0.01	0.49±0.01	**0.55±0.01**	0.51±0.01	0.43±0.01
Sensitivity (m°C/mM)	0.0723	0.0753	0.0770	**0.0810**	0.0763	0.0730
R^2	0.9951	0.09986	0.9961	**0.9997**	0.9949	0.9941
Analysis time (min)	8-9	8-9	8-9	**8-9**	8-10	8-10



(a)



(b)

Figure 36 Responses of the glucose calorimetric biosensor system using different buffer concentration (a) enzyme reactor column with immobilized glucose oxidase (b) enzyme reactor column with glucose oxidase co-immobilized catalase

3.3.1.4 pH of buffer

Responses from both enzyme reactor columns using phosphate buffer at pH 6.00, 6.50, 6.80, 7.00, 7.20, 7.50 and 8.00 were shown in Table 25 and Figure 37. In both enzyme reactor columns the pH 7.00 gave maximum response and phosphate buffer pH 7.00 was chosen for further work.

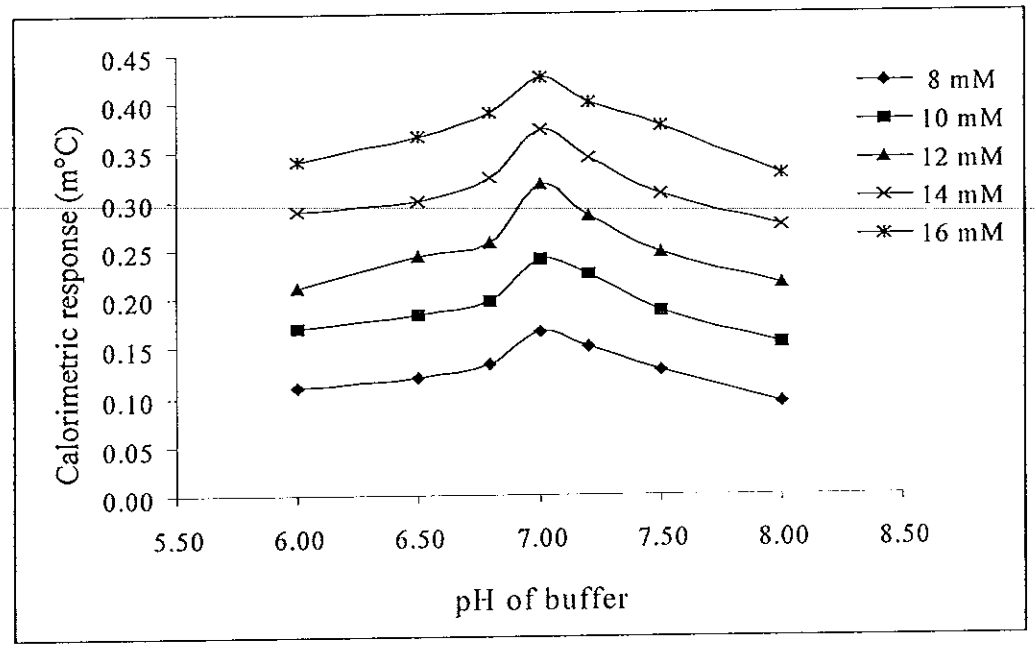
Table 25 Responses of the glucose calorimetric biosensor system using different pH of buffer

(a) immobilized glucose oxidase

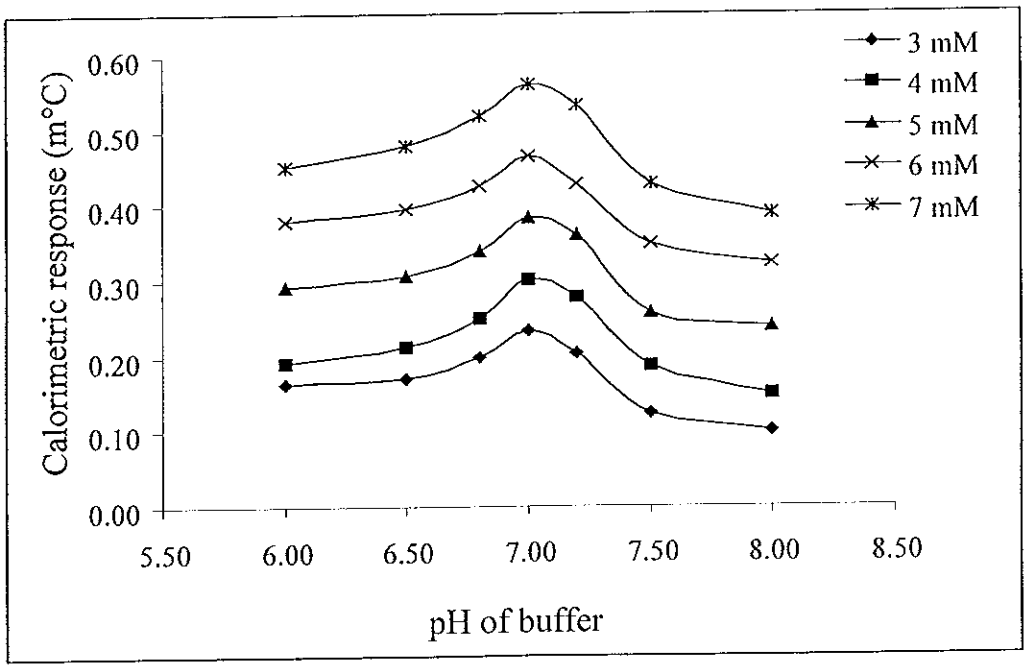
Glucose concentration (mM)	Amperometric response (m°C), different pH of buffer						
	6.00	6.50	6.80	**7.00**	7.20	7.50	8.00
	Mean±SD	Mean±SD	Mean±SD	**Mean±SD**	Mean±SD	Mean±SD	Mean±SD
8.0	0.11±0.01	0.12±0.01	0.13±0.01	**0.17±0.01**	0.15±0.01	0.13±0.01	0.09±0.02
10	0.17±0.01	0.18±0.01	0.20±0.01	**0.24±0.01**	0.22±0.01	0.19±0.01	0.15±0.01
12	0.21±0.01	0.24±0.01	0.26±0.01	**0.32±0.01**	0.28±0.01	0.25±0.01	0.21±0.01
14	0.29±0.01	0.30±0.02	0.32±0.01	**0.37±0.01**	0.34±0.01	0.31±0.01	0.27±0.01
16	0.34±0.01	0.37±0.01	0.39±0.01	**0.43±0.01**	0.40±0.01	0.38±0.02	0.33±0.01
Sensitivity (m°C/mM)	0.0290	0.0305	0.0320	**0.0327**	0.0310	0.0310	0.0293
R^2	0.9917	0.9994	0.9996	**0.9934**	0.9976	0.999	0.9995
Analysis time(min)	8-9	8-9	8-9	**8-9**	8-9	8-9	8-9

(b) glucose oxidase co-immobilized with catalase

Glucose concentration (mM)	Amperometric response (m°C), different pH of concentration						
	6.00	6.50	6.80	**7.00**	7.20	7.50	8.00
	Mean±SD	Mean±SD	Mean±SD	**Mean±SD**	Mean±SD	Mean±SD	Mean±SD
3.0	0.16±0.01	0.17±0.01	0.20±0.00	**0.23±0.01**	0.20±0.02	0.12±0.01	0.10±0.00
4.0	0.19±0.01	0.21±0.01	0.25±0.01	**0.30±0.01**	0.28±0.00	0.19±0.01	0.15±0.01
5.0	0.29±0.01	0.31±0.01	0.34±0.01	**0.38±0.01**	0.36±0.01	0.26±0.01	0.24±0.00
6.0	0.38±0.00	0.40±0.02	0.43±0.01	**0.047±0.01**	0.43±0.01	0.35±0.01	0.32±0.01
7.0	0.45±0.01	0.48±0.00	0.52±0.00	**0.56±0.01**	0.53±0.01	0.43±0.01	0.39±0.01
Sensitivity (m°C/mM)	0.0767	0.0803	0.0817	**0.0823**	0.0810	0.0777	0.0753
R^2	0.9804	0.9881	0.9904	**0.9966**	0.9959	0.9949	0.9932
Analysis time(min)	8-9	8-9	8-9	**8-9**	8-9	8-9	8-9



(a)



(b)

Figure 37 Responses of the glucose calorimetric biosensor system using different pH of buffer (a) enzyme reactor column with immobilized glucose oxidase (b) enzyme reactor column with glucose oxidase co-immobilized catalase

3.3.1.5 Effect of 0.9%(w/v) NaCl

The effect of 0.9%(w/v) NaCl on the calorimetric system response was investigated using the same procedure as in the amperometric system (2.10.1.6). That is (I) no salt in any solution, (II) salt was added to standard glucose solution and (III) salt was added to standard glucose solution and buffer of sample line. The responses of the enzyme reactor columns of standard glucose solution are shown in Table 26 and Figure 38. In case (II) of both reactor columns the peak height is higher than others. This higher response was due to the increase of heat from the oxidation reaction of glucose and the heat generated by the increase in the ionic strength (McLean and Penketh, 1968) from the diffusion of salt (NaCl) in the sample line through the dialysis membrane into the buffer line which had not salt. For cases (I) and (III) the peak height were similar because the ionic strength on both sides of the dialyser were equal and the response were only from the oxidation of glucose. Condition (III) was then chosen since the added salt in the standard glucose solution and buffer of sample line will provide an isotonic condition for the plasma samples.

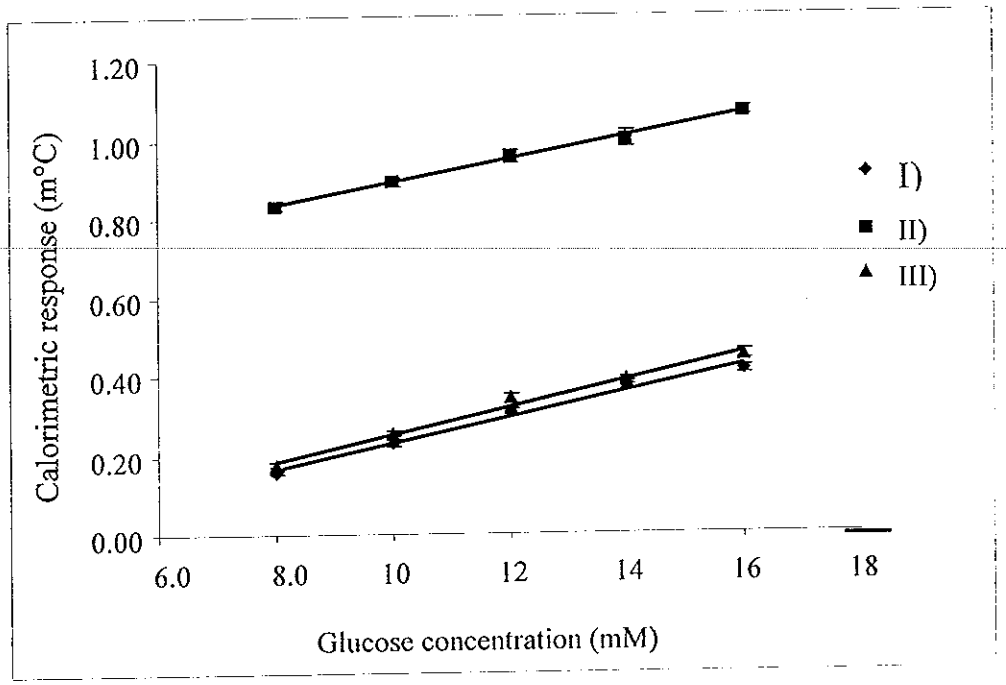
Table 26 Responses of the glucose calorimetric biosensor system on the effect of 0.9%(w/v) NaCl

(a) immobilized glucose oxidase

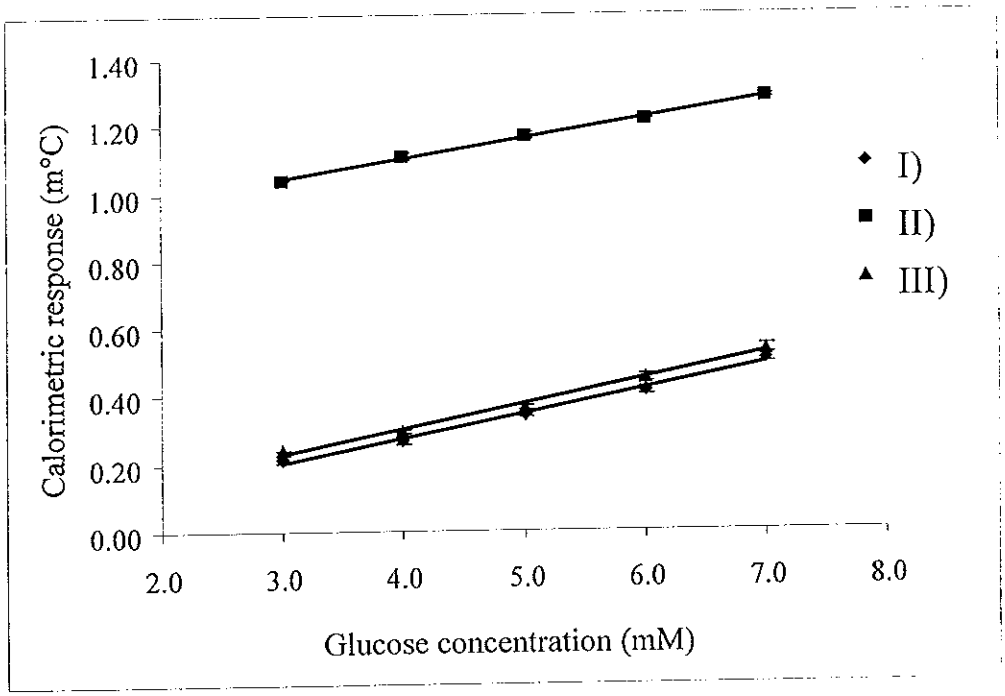
Glucose concentration (mM)	Amperometric response (mV), effect of 0.9%(w/v) NaCl at condition		
	I	II	**III**
	Mean±SD	Mean±SD	**Mean±SD**
8	0.16±0.01	0.83±0.01	**0.18±0.01**
10	0.23±0.01	0.89±0.01	**0.25±0.01**
12	0.31±0.01	0.96±0.02	**0.35±0.01**
14	0.37±0.01	1.00±0.02	**0.39±0.01**
16	0.41±0.01	1.07±0.01	**0.45±0.01**
Sensitivity (m°C/mM)	0.0322	0.0287	**0.0337**
R^2	0.9889	0.9968	**0.9883**
Analysis time (min)	8-9	8-9	**8-9**

(b) glucose oxidase co-immobilized with catalase

Glucose concentration (mM)	Amperometric response (mV), effect of 0.9%(w/v) NaCl at condition		
	I	II	**III**
	Mean±SD	Mean±SD	**Mean±SD**
3	0.21±0.01	1.04±0.00	**0.24±0.00**
4	0.27±0.01	1.11±0.01	**0.30±0.01**
5	0.34±0.01	1.17±0.01	**0.37±0.01**
6	0.41±0.01	1.22±0.00	**0.45±0.01**
7	0.51±0.01	1.28±0.01	**0.53±0.01**
Sensitivity (m°C/mM)	0.0730	0.0594	**0.0740**
R^2	0.9928	0.9972	**0.9939**
Analysis time (min)	8-9	8-9	**8-9**



(a)



(b)

Figure 38 Responses of the glucose calorimetric biosensor system on the effect of 0.9%(w/v) NaCl (a) enzyme reactor column with immobilized glucose (b) enzyme reactor column with glucose oxidase co-immobilized catalase

3.3.1.6 Sample volume

The responses of standard glucose solution at different sample volume *i.e.* 200, 300, 400, 500 and 600 µl pulsed into the system are shown in Table 27 and Figure 39. The response and the analysis time increased as the sample volume increased with the sensitivity highest at 600 µl. However, the analysis time was too long. Therefore , the sample volume of 500 µl was chosen because the sensitivity was only slightly lower than 600µl, 1.92% for the reactor column with immobilized glucose oxidase and 0.60% for the reactor column with glucose oxidase co-immobilized catalase, but the analysis time was shorter and this was appropriated to analysis in plasma sample.

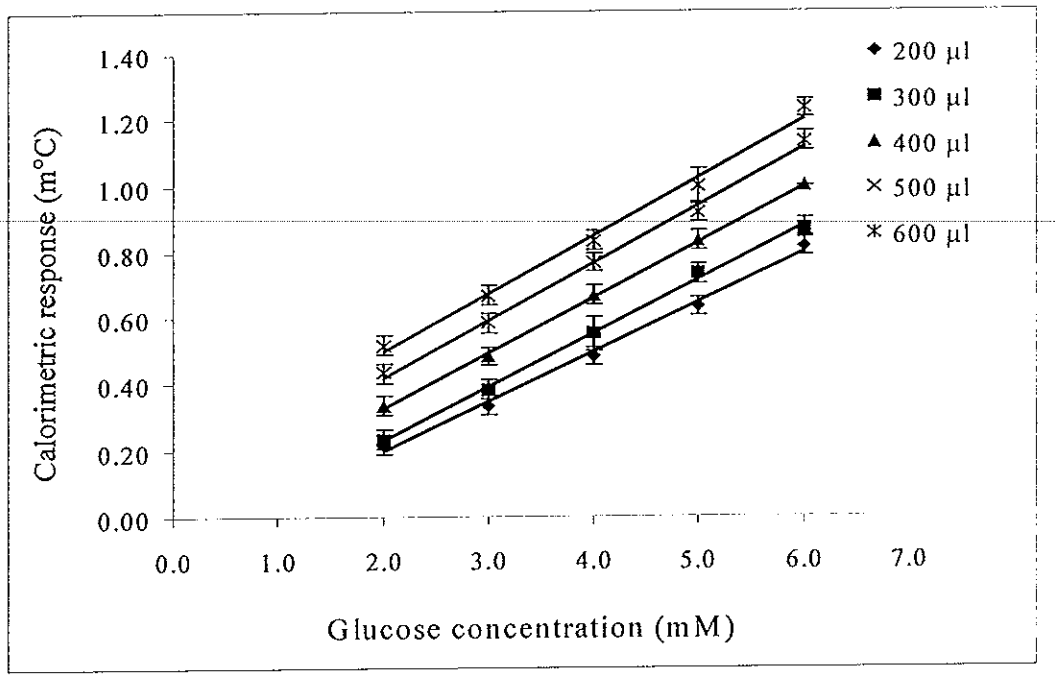
Table 27 Responses of the glucose calorimetric biosensor system using different sample volume

(a) immobilized glucose oxidase

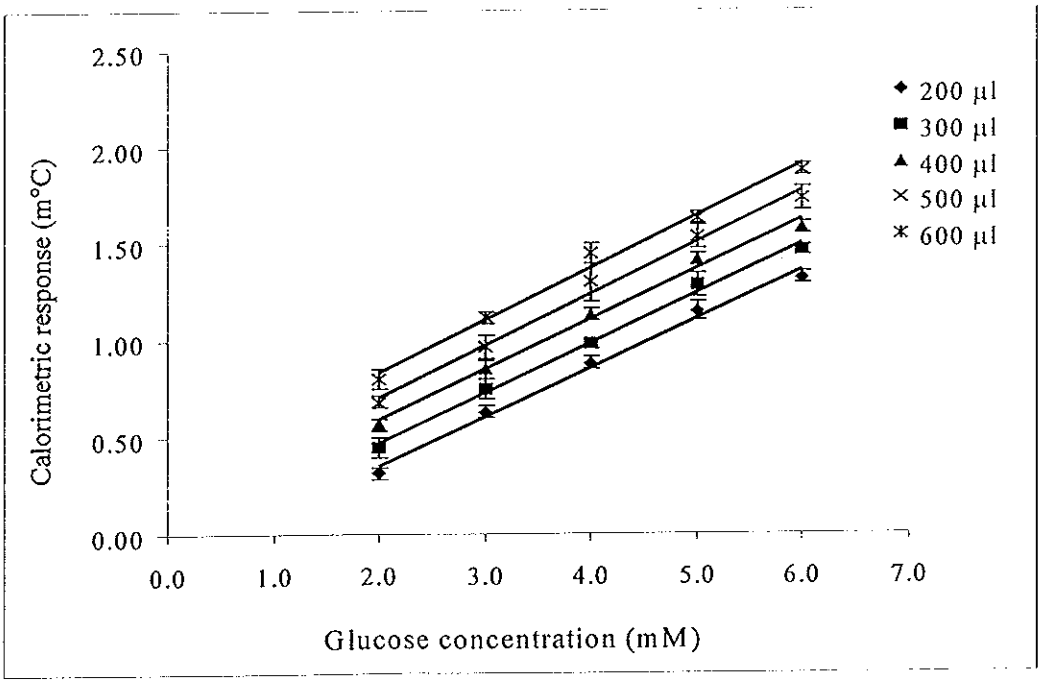
Glucose concentration (mM)	Calorimetric response (m°C), different sample volume				
	200 µl	300 µl	400 µl	**500 µl**	600 µl
	Mean ± SD	Mean ± SD	Mean ± SD	**Mean ± SD**	Mean ± SD
2.0	0.22±0.03	0.23±0.03	0.33±0.03	**0.43±0.03**	0.52±0.03
3.0	0.33±0.03	0.38±0.03	0.48±0.03	**0.58±0.03**	0.67±0.03
4.0	0.48±0.03	0.55±0.05	0.67±0.03	**0.77±0.03**	0.83±0.03
5.0	0.63±0.03	0.73±0.03	0.83±0.03	**0.92±0.03**	1.00±0.05
6.0	0.82±0.03	0.87±0.03	1.00±0.00	**1.13±0.03**	1.23±0.03
Sensitivity (m°C/mM)	0.1500	0.1623	0.1683	**0.1733**	0.1767
R^2	0.9941	0.9982	0.9993	**0.9959**	0.9926
Analysis time (min)	7-8	7-8	8-9	**8-9**	10-11

(b) glucose oxidase co-immobilized with catalase

Glucose concentration (mM)	Calorimetric response (m°C), different sample volume				
	200 µl	300 µl	400 µl	**500 µl**	600 µl
	Mean ± SD	Mean ± SD	Mean ± SD	**Mean ± SD**	Mean ± SD
2.0	0.32±0.03	0.45±0.05	0.57±0.03	**0.68±0.03**	0.80±0.05
3.0	0.63±0.03	0.75±0.05	0.85±0.05	**0.97±0.06**	1.12±0.03
4.0	0.88±0.03	0.98±0.03	1.13±0.03	**1.30±0.10**	1.45±0.05
5.0	1.15±0.05	1.28±0.06	1.42±0.03	**1.53±0.06**	1.63±0.03
6.0	1.32±0.03	1.47±0.03	1.58±0.03	**1.73±0.06**	1.88±0.03
Sensitivity (m°C/mM)	0.2523	0.2567	0.2593	**0.2667**	0.2683
R^2	0.9905	0.9945	0.9915	**0.9910**	0.9889
Analysis time (min)	7-8	7-8	8-9	**8-9**	10-11



(a)



(b)

Figure 39 Responses of the glucose calorimetric biosensor system using different sample volume (a) enzyme reactor column with immobilized glucose oxidase (b) enzyme reactor column with glucose oxidase co-immobilized catalase

3.3.2 Flow injection system

3.3.2.1 Optimization of the flow rate

a) Flow rate of sample line

The responses of the enzyme reactor columns at different flow rates of the sample line are shown in Table 28 and Figure 40. For both reactors the highest signals were obtained at the slowest flow rate, 0.2 ml min^{-1}. However, the analysis time was much longer than others. At 0.4 ml min^{-1} the sensitivity was only 9.72% lower for the reactor column with immobilized glucose oxidase and 1.80% lower for the reactor column with glucose oxidase co-immobilized catalase than 0.2 ml min^{-1} but the analysis time was much shorter (9 min compared to 13 min). So, it was chosen to be used in further analysis.

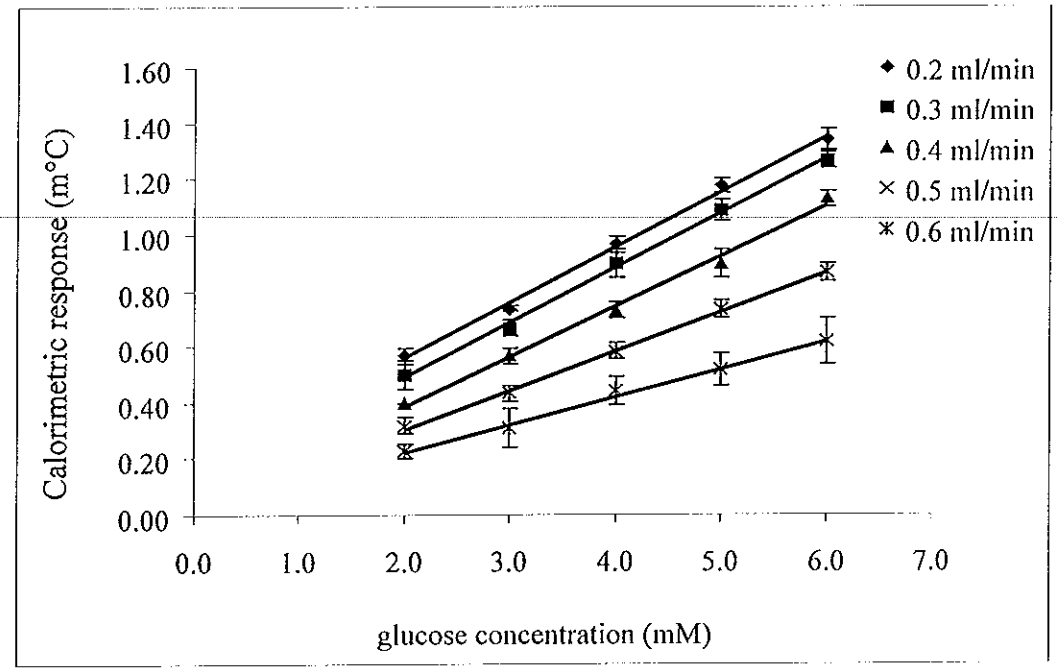
Table 28 Responses of theflow-injection calorimetric biosensor system using different flow rate of the sample line with buffer line flow rate 0.75 ml min^{-1}

(a) immobilized glucose oxidase

Glucose concentration (mM)	Calorimetric response (m°C), different flow rate of sample line				
	0.20 ml/min	0.30 ml/min	**0.40 ml/min**	0.50 ml/min	0.60 ml/min
	Mean ± SD	Mean ± SD	**Mean ± SD**	Mean ± SD	Mean ± SD
2.0	0.57±0.03	0.50±0.05	**0.40±0.00**	0.32±0.03	0.23±0.03
3.0	0.74±0.01	0.67±0.03	**0.57±0.03**	0.43±0.03	0.31±0.07
4.0	0.97±0.03	0.90±0.05	**0.73±0.05**	0.58±0.03	0.44±0.05
5.0	1.17±0.03	1.09±0.04	**0.90±0.05**	0.73±0.03	0.52±0.06
6.0	1.34±0.04	1.27±0.03	**1.13±0.03**	0.87±0.03	0.62±0.08
Sensitivity (m°C/mM)	0.1986	0.1957	**0.1793**	0.1400	0.1039
R^2	0.99720	0.9975	**0.9947**	0.9983	0.9939
Analysis time (min)	11-13	9-11	**8-9**	8-9	8-9

(b) glucose oxidase co-immobilized with catalase

Glucose concentration (mM)	Calorimetric response (m°C), different flow rate of sample line				
	0.20 ml/min	0.30 ml/min	**0.40 ml/min**	0.50 ml/min	0.60 ml/min
	Mean ± SD	Mean ± SD	**Mean ± SD**	Mean ± SD	Mean ± SD
2.0	0.80±0.00	0.73±0.03	**0.67±0.03**	0.60±0.05	0.53±0.03
3.0	1.13±0.03	1.07±0.03	**0.97±0.06**	0.87±0.03	0.77±0.03
4.0	1.43±0.03	1.33±0.03	**1.27±0.03**	1.15±0.05	1.08±0.03
5.0	1.68±0.03	1.62±0.03	**1.53±0.06**	1.42±0.03	1.30±0.00
6.0	1.92±0.03	1.83±0.03	**1.75±0.05**	1.67±0.03	1.58±0.03
Sensitivity (m°C/mM)	0.2783	0.2743	**0.2733**	0.2683	0.2633
R^2	0.9942	0.9949	**0.9956**	0.9996	0.9975
Analysis time (min)	11-13	9-11	**8-9**	8-9	8-9



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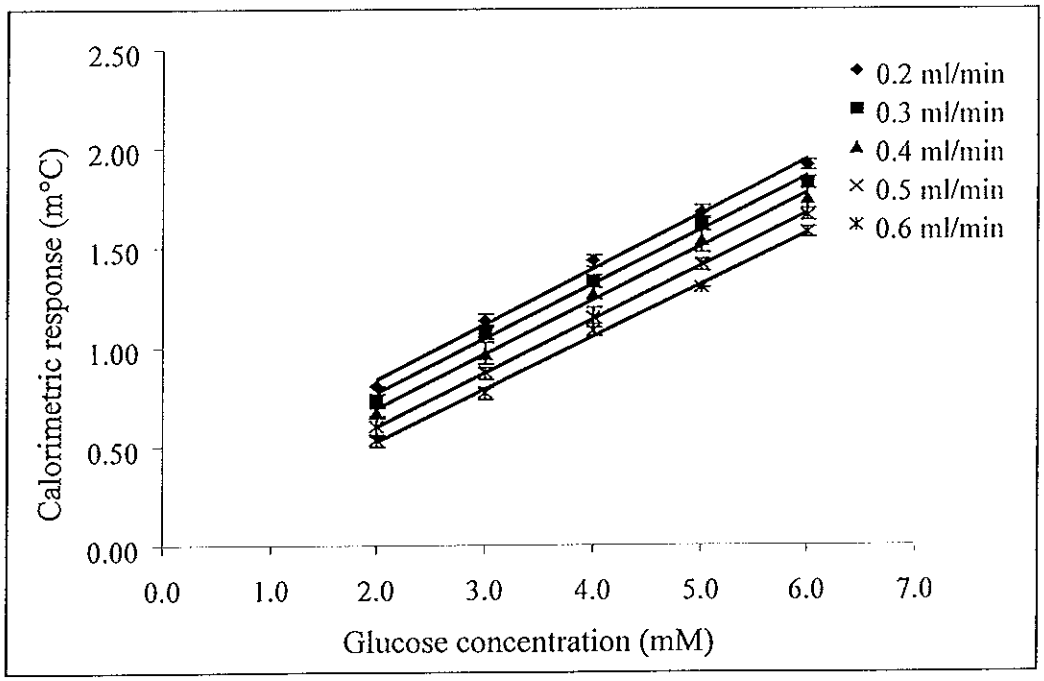


(b)

Figure 40 Responses of the flow-injection calorimetric biosensor system using different flow rate of sample line (a) enzyme reactor column with immobilized glucose oxidase (b) enzyme reactor column with glucose oxidase co-immobilized catalase

b) Flow rate of buffer line

Table 29 and Figure 41 shown the effect of the flow rate of buffer line on the calorimetric system for the two enzyme reactor columns with immobilized glucose oxidase and glucose oxidase co-immobilized with catalase. For both reactor columns, the peak heights and the sensitivity increases as the flow rate increased. The slowest flow rate, 0.4 ml min^{-1}, gave the highest sensitivity but also gave the longest analysis time (23-25 min). In this case the flow rate of 0.75 ml min^{-1} was chosen. Although its sensitivity was lower than at 0.40 ml min^{-1} but it was still quite high and its analysis time was much shorter.

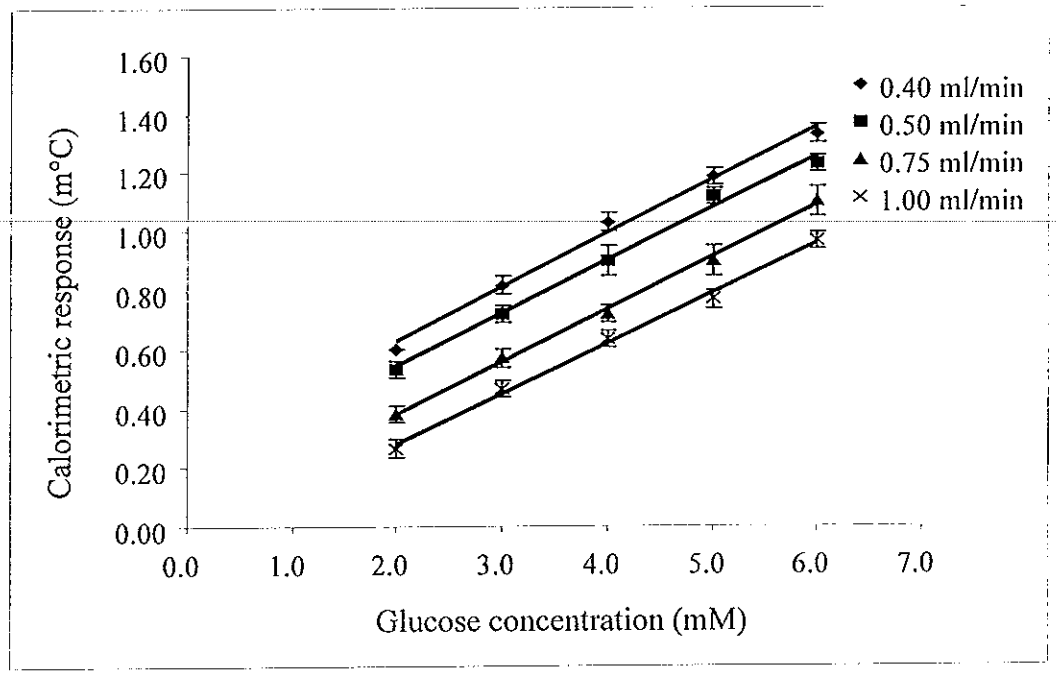
Table 29 Responses of the flow-injection calorimetric biosensor system using different flow rate of buffer line with sample line flow rate 0.40 ml min^{-1}

(a) immobilized glucose oxidase

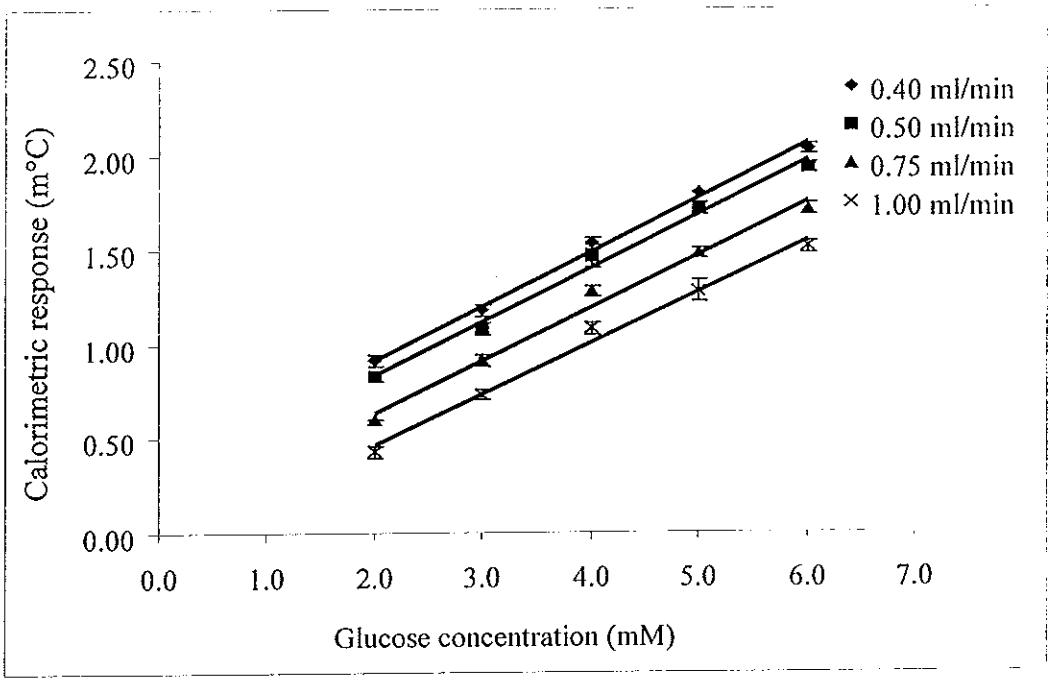
Glucose concentration (mM)	Calorimetric response (m°C), different flow rate of buffer line			
	0.40 ml/min	0.50 ml/min	**0.75 ml/min**	1.00 ml/min
	Mean ± SD	Mean ± SD	**Mean ± SD**	Mean ± SD
2.0	0.60±0.00	0.53±0.03	**0.38±0.03**	0.27±0.03
3.0	0.82±0.03	0.72±0.03	**0.57±0.03**	0.47±0.03
4.0	1.03±0.03	0.90±0.05	**0.72±0.03**	0.63±0.03
5.0	1.18±0.03	1.12±0.03	**0.90±0.05**	0.77±0.03
6.0	1.33±0.03	1.23±0.03	**1.10±0.05**	0.97±0.03
Sensitivity (m°C/mM)	0.1833	0.1790	**0.1770**	0.1700
R^2	0.9916	0.9929	**0.9980**	0.9958
Analysis time (min)	23-25	18-20	**8-9**	7-8

(b) glucose oxidase co-immobilized with catalase

Glucose concentration (mM)	Calorimetric response (m°C), different flow rate of buffer line			
	0.40 ml/min	0.50 ml/min	**0.75 ml/min**	1.00 ml/min
	Mean ± SD	Mean ± SD	**Mean ± SD**	Mean ± SD
2.0	0.92±0.03	0.83±0.03	**0.60±0.00**	0.43±0.03
3.0	1.18±0.03	1.08±0.03	**0.92±0.03**	0.73±0.03
4.0	1.53±0.03	1.47±0.06	**1.28±0.03**	1.08±0.03
5.0	1.80±0.00	1.72±0.03	**1.48±0.03**	1.28±0.06
6.0	2.03±0.03	1.93±0.03	**1.72±0.03**	1.52±0.03
Sensitivity (m°C/mM)	0.2853	0.2843	**0.2800**	0.2733
R^2	0.9953	0.9905	**0.9875**	0.9892
Analysis time (min)	23-25	18-20	**8-9**	7-8



(a)



(b)

Figure 41 Responses of the flow-injection calorimetric biosensor system using different flow rate of buffer line (a) enzyme reactor column with immobilized glucose oxidase (b) enzyme reactor column with glucose oxidase co-immobilized catalase

3.3.2.2 Sample volume

The effect of the sample volume, 200 to 500 µl, of standard glucose solution are shown in Table 30 and Figure 42. In both enzyme reactor columns, the peak heights and the sensitivities increased as the sample volume increased. If the sample volume was further increased the response would probably increase, but the analysis time would also increase. Therefore, the sample volume of 500 µl was chosen because of the sensitivity was higher than others while the analysis time was not much different.

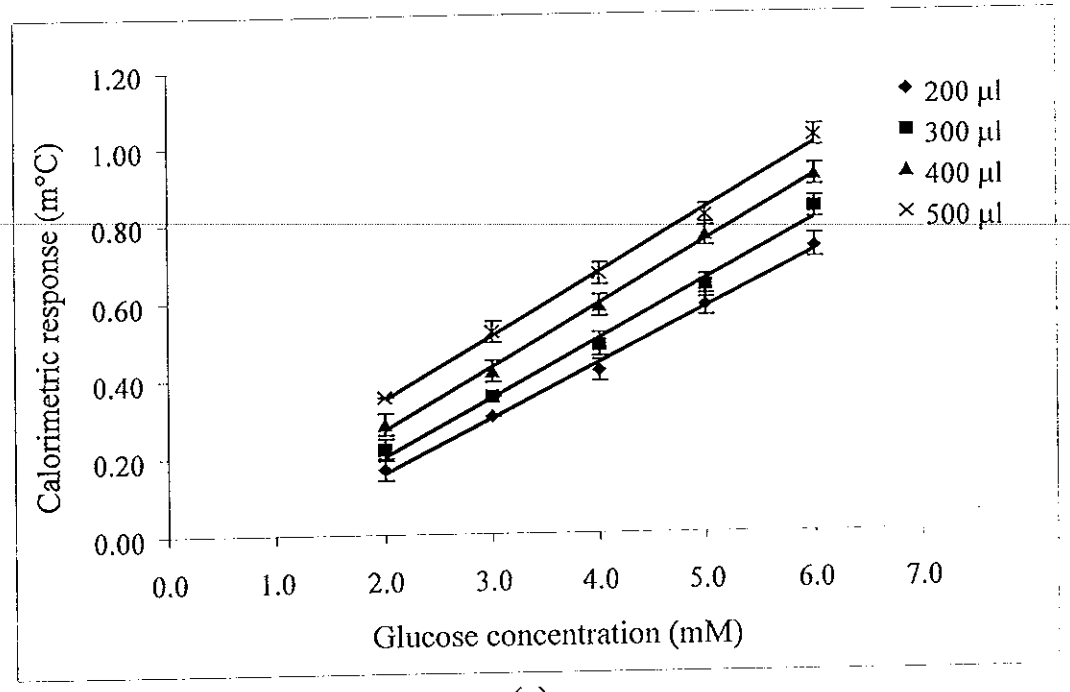
Table 30 Responses of the flow-injection calorimetric biosensor system using different sample volume

(a) immobilized glucose oxidase

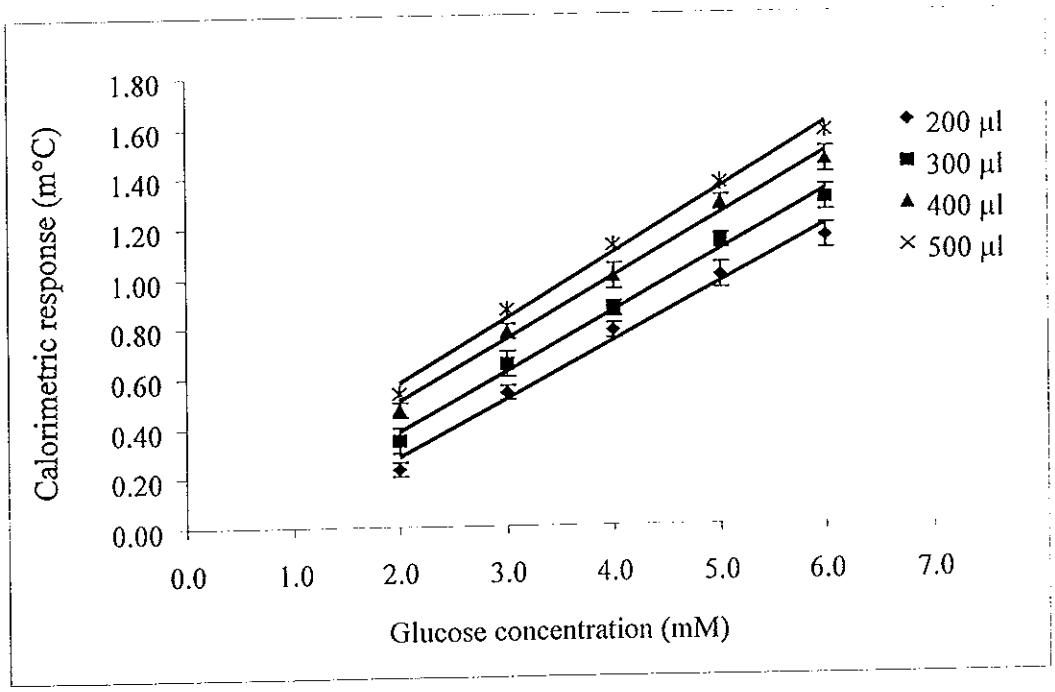
Glucose concentration (mM)	Calorimetric response (m°C), different sample volume			
	200 µl	300 µl	400 µl	**500 µl**
	Mean ± SD	Mean ± SD	Mean ± SD	**Mean ± SD**
2.0	0.17±0.03	0.22±0.03	0.28±0.03	**0.35±0.00**
3.0	0.30±0.00	0.35±0.00	0.42±0.03	**0.52±0.03**
4.0	0.42±0.03	0.48±0.03	0.58±0.03	**0.67±0.03**
5.0	0.58±0.03	0.63±0.03	0.77±0.03	**0.82±0.03**
6.0	0.73±0.03	0.83±0.03	0.92±0.03	**1.02±0.03**
Sensitivity (m°C/mM)	0.1417	0.1517	0.1617	**0.1637**
R^2	0.9965	0.9976	0.9920	**0.9963**
Analysis time (min)	6-7	7-8	8-9	**8-9**

(b) glucose oxidase co-immobilized with catalase

Glucose concentration (mM)	Calorimetric response (m°C), different sample volume			
	200 µl	300 µl	400 µl	**500 µl**
	Mean ± SD	Mean ± SD	Mean ± SD	**Mean ± SD**
2.0	0.23±0.03	0.35±0.05	0.47±0.03	**0.53±0.03**
3.0	0.53±0.03	0.65±0.05	0.78±0.03	**0.87±0.03**
4.0	0.78±0.03	0.87±0.03	1.00±0.05	**1.12±0.03**
5.0	1.00±0.05	1.13±0.03	1.28±0.03	**1.37±0.03**
6.0	1.15±0.05	1.30±0.05	1.45±0.05	**1.57±0.03**
Sensitivity (m°C/mM)	0.2300	0.2383	0.2467	**0.2567**
R^2	0.9915	0.9908	0.9921	**0.9851**
Analysis time (min)	6-7	7-8	8-9	**8-9**



(a)



(b)

Figure 42 Responses of the flow-injection calorimetric biosensor system using different sample volume (a) enzyme reactor column with immobilized glucose oxidase (b) enzyme reactor column with glucose oxidase co-immobilized catalase

3.3.3 Comparison of the flow-through and flow-injection system.

Both enzyme reactor columns were tested by the two proposed systems at flow rate of the buffer and the sample lines 0.75 and 0.4 ml min^{-1}, respectively, and a sample volume of 500 µl. The results are shown in Table 31 and Figure 43. The system with two enzymes provided lower detection limit than when only one enzyme was used, 0.1 mM and 0.3 mM, respectively. Both enzyme reactor columns had similar analysis time between 8-9 min. However, the sensitivity of the flow injection system was slightly higher than the flow-through system and the linearity was better. These were the reasons that the flow-injection system was used for this work.

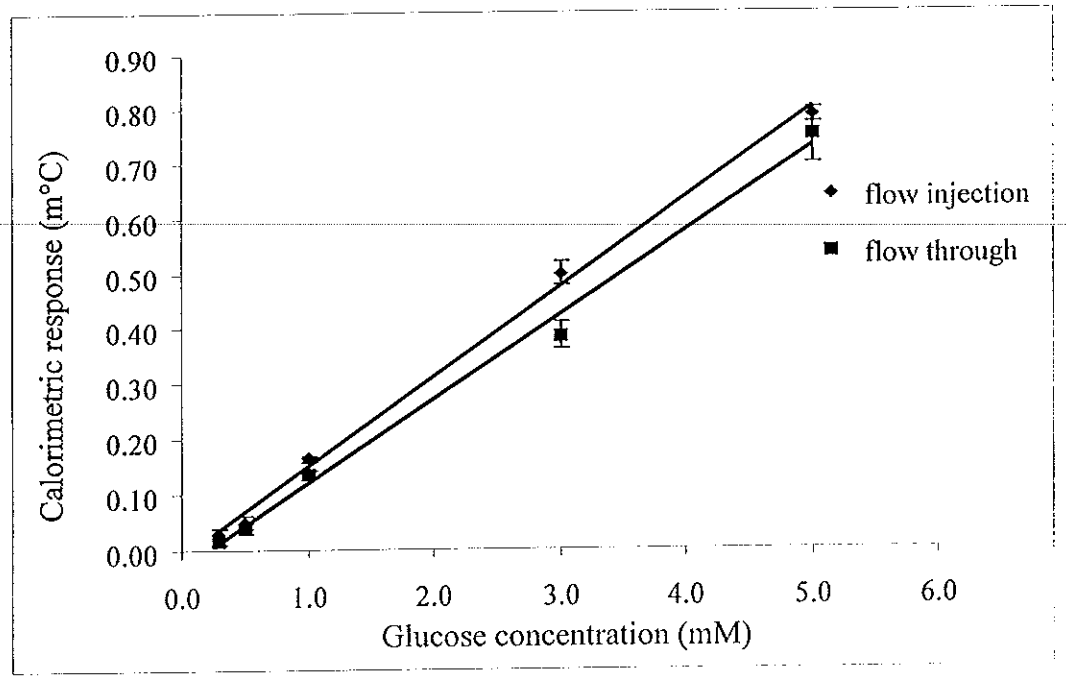
Table 31 Comparison of the response of the flow-through and flow-injection amperometric biosensor system

(a) immobilized glucose oxidase

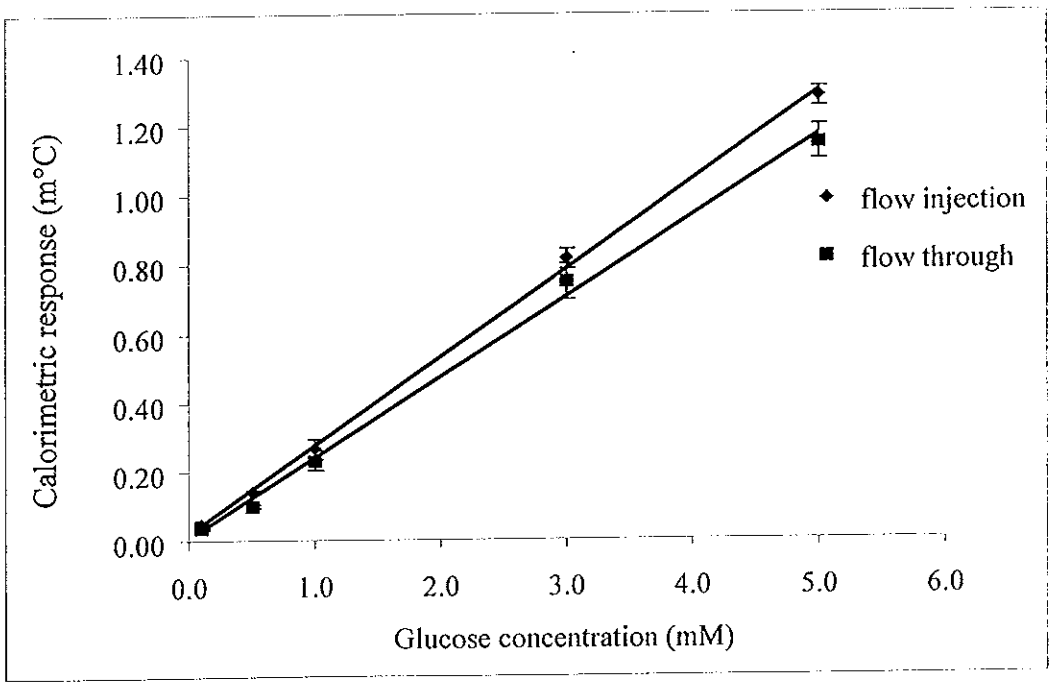
Glucose concentration (mM)	Calorimetric response (m°C)	
	Flow –through system	Flow-injection system
	Mean±SD	**Mean±SD**
0.3	0.02±0.01	**0.03±0.01**
0.5	0.04±0.01	**0.05±0.01**
1.0	0.14±0.01	**0.16±0.01**
3.0	0.39±0.02	**0.50±0.02**
5.0	0.75±0.05	**0.79±0.01**
Sensitivity (m°C/mM)	0.1528	**0.1631**
R^2	0.9943	**0.9967**
Max. %RSD	50.0	**33.3**
Analysis time (min)	8-9	**8-9**

(b) glucose oxidase co-immobilized with catalase

Glucose concentration (mM)	Calorimetric response (m°C)	
	Flow–through system	Flow-injection system
	Mean±SD	**Mean±SD**
0.1	0.04±0.01	**0.05±0.01**
0.5	0.10±0.01	**0.14±0.01**
1.0	0.23±0.03	**0.27±0.03**
3.0	0.75±0.05	**0.82±0.03**
5.0	1.15±0.05	**1.28±0.03**
Sensitivity (m°C/mM)	0.2335	**0.2556**
R^2	0.9967	**0.9989**
Max. %RSD	25.0	**20.0**
Analysis time (min)	8-9	**8-9**



(a)



(b)

Figure 43 Comparison of the response of the flow-through and flow-injection calorimetric biosensor system (a) enzyme reactor column with immobilized glucose oxidase (b) enzyme reactor column with glucose oxidase co-immobilized catalase

3.3.4 Stability of the enzyme reactor column

The long term stability of the immobilized enzyme used in the calorimetric system was tested intermittently over a 2 month period, 210 h operation time for the reactor column with immobilized glucose oxidase and 203 h operation time for the reactor column with glucose oxidase co-immobilized catalase, by monitor its response to 500 µl of standard glucose solutions. The responses are shown in Table 32. The sensitivity of the two enzyme reactor columns gradually decreased as the operation time increased (Figure 44).

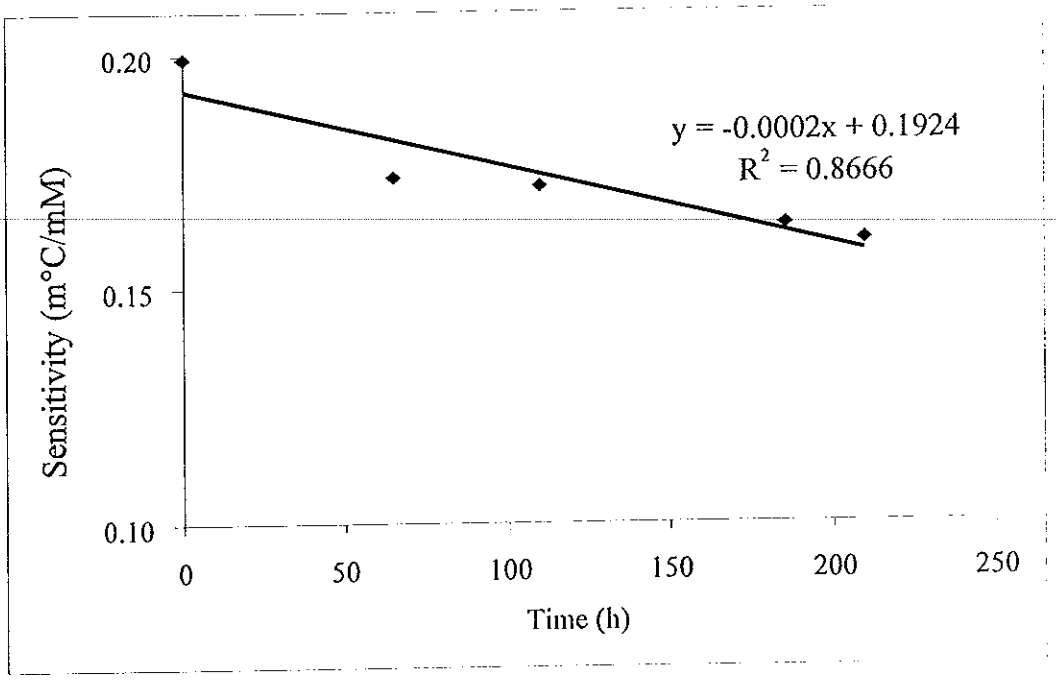
Table 32 Responses of the calorimetric biosensor system at different operation time of the enzyme reactor

(a) immobilized glucose oxidase

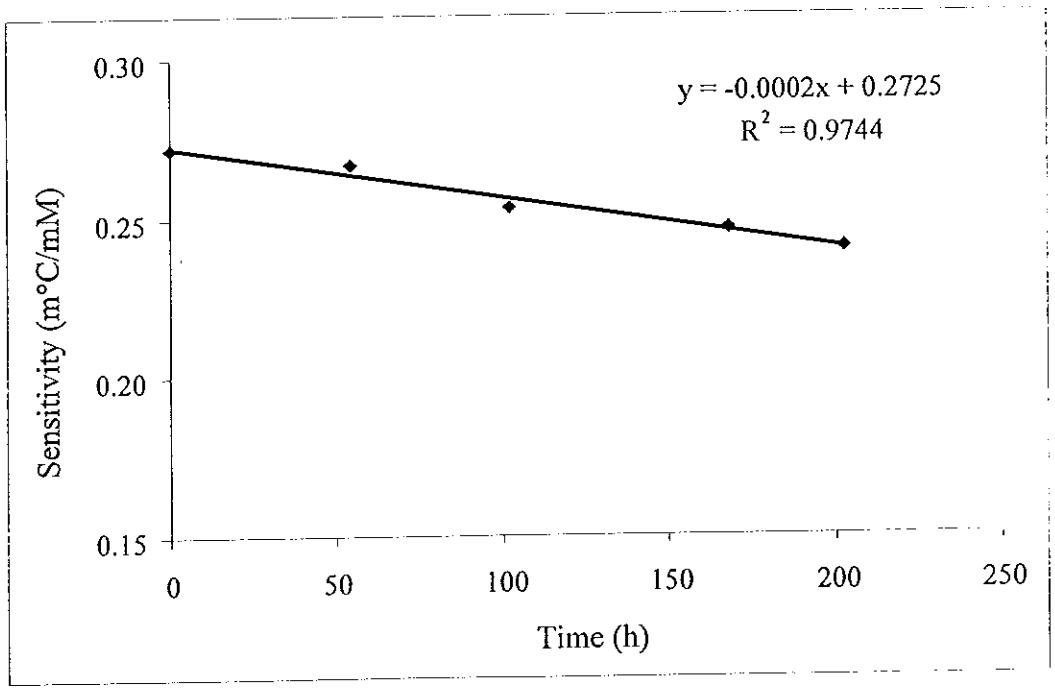
Glucose concentration (mM)	Calorimetric response (m°C), operation time (h)				
	0 h	65 h	110 h	185 h	210 h
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
2.0	0.50 ±0.05	0.43 ±0.03	0.38 ±0.03	0.35 ±0.00	0.28 ±0.03
3.0	0.67 ±0.03	0.58 ±0.03	0.57 ±.0.03	0.52 ±0.03	0.39 ±0.02
4.0	0.90 ±0.05	0.77 ±0.03	0.72 ±0.03	0.67 ±0.03	0.60 ±0.05
5.0	1.12 ±0.03	0.92 ±0.03	0.85 ±0.05	0.82 ±0.03	0.75 ±0.05
6.0	1.27 ±0.03	1.13 ±0.03	1.10 ±0.05	1.02 ±0.03	0.90 ±0.03
Sensitivity (m°C/mM)	0.1991	0.1733	0.1717	0.1633	0.1597
R^2	0.9938	0.9959	0.9885	0.9971	0.9919
Analysis time (min)	8-9	8-9	8-9	8-9	8-9

(b) glucose oxidase co-immobilized with catalase

Glucose concentration (mM)	Calorimetric response (m°C), operation time (h)				
	0 h	54 h	102 h	168 h	203 h
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
2.0	0.87±0.03	0.68±0.03	0.60±0.00	0.53±0.03	0.43±0.08
3.0	1.23±0.03	0.97±0.06	0.92±0.03	0.83±.0.03	0.75±0.05
4.0	1.52±0.03	1.30±0.10	1.22±0.03	1.12±.0.06	0.97±0.03
5.0	1.75±0.05	1.53±0.06	1.42±0.03	1.37±0.03	1.15±0.05
6.0	1.97±0.03	1.73±0.06	1.62±0.03	1.50±0.03	1.43±0.03
Sensitivity (m°C/mM)	0.2717	0.2667	0.2533	0.2467	0.2400
R^2	0.9877	0.9910	0.9870	0.9828	0.9918
Analysis time (min)	8-9	8-9	8-9	8-9	8-9



(a)



(b)

Figure 44 Sensitivity of the calorimetric biosensor system at different operation time of enzyme reactor (a) enzyme reactor column with immobilized glucose oxidase (b) enzyme reactor column with glucose oxidase co-immobilized catalase

3.3.5 Linearity of the responses in the calorimetric biosenosr system

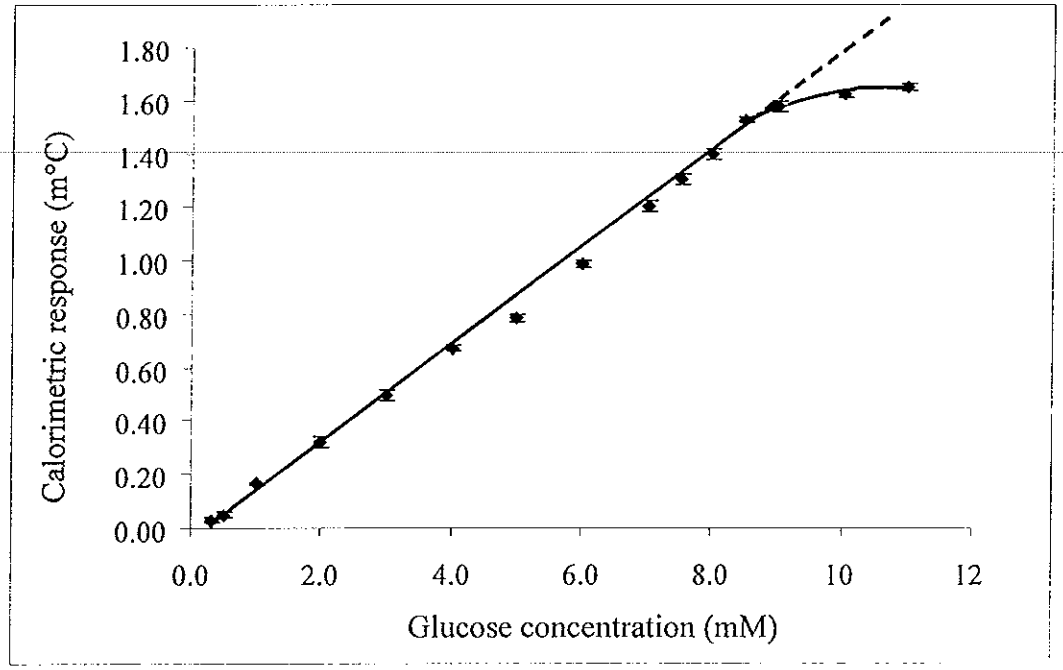
The linearity of the response of the system to different concentration of standard glucose solution are shown in Table 33 and Figure 45. The linear range of the reactor column with immobilized glucose oxidase and the reactor column with glucose oxidase co-immobilized catalase were 0.3-8.5 mM and 0.1-8.5 mM respectively.

Table 33 Response of the calorimetric system to different concentration of glucose (ND= non detectable)

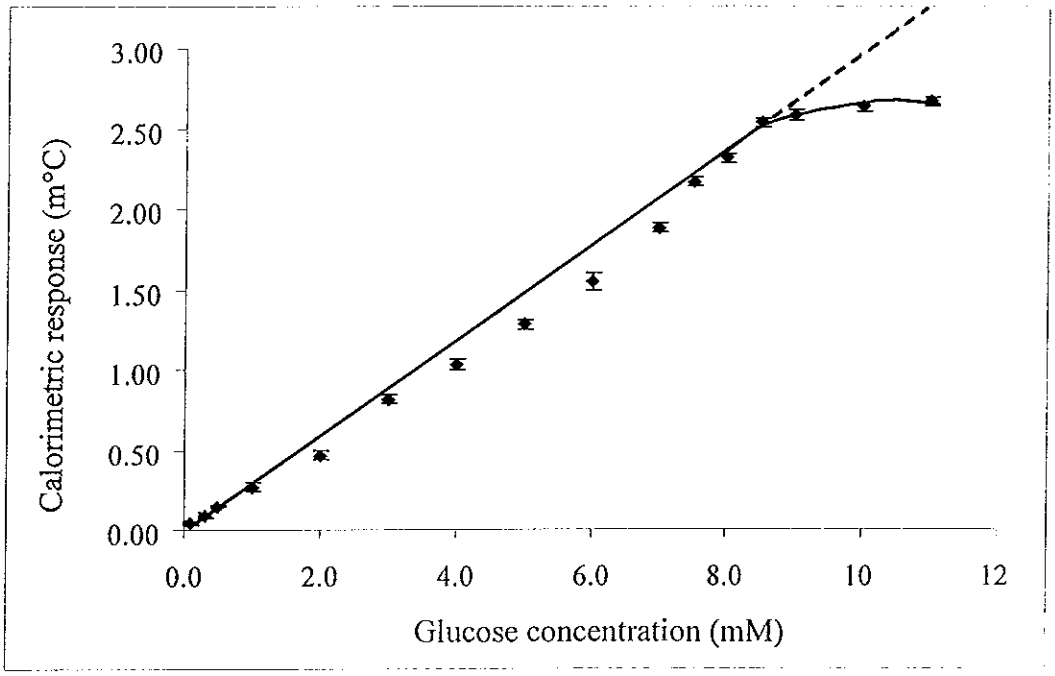
(a) immobilized glucose oxidase

(b) glucose oxidase co-immobilized with catalase

Concentraion of glucose (mM)	Response (m°C) Mean ± SD	
	(a)	(b)
0.1	ND	0.05±0.01
0.3	0.03±0.01	0.09±0.01
0.5	0.05±0.01	0.14±0.01
1.0	0.16±0.01	0.27±0.03
2.0	0.32±0.02	0.47±0.03
3.0	0.50±0.02	0.82±0.03
4.0	0.67±0.01	1.03±0.03
5.0	0.79±0.01	1.28±0.03
6.0	0.99±0.01	1.55±0.05
7.0	1.20±0.02	1.88±0.03
7.5	1.30±0.02	2.17±0.03
8.0	1.40±0.02	2.32±0.03
8.5	1.53±0.01	2.53±0.03
9.0	1.58±0.02	2.58±0.03
10.0	1.63±0.01	2.63±0.03
11.0	1.65±0.02	2.67±0.03
Sensitivity (m°C/mM) of 0.3-8.5 mM glucose standard solution	0.1179	0.2865
R^2	0.9969	0.9919
Analysis time (min)	8-10	8-10



(a)



(b)

Figure 45 Response of the system to different concentration of glucose in the calorimetric biosensor system a) enzyme reactor column with immobilized glucose oxidase (b) enzyme reactor column with glucose oxidase co-immobilized catalase

3.4 Determination of glucose in plasma samples

The measurement of glucose using the two biosensor systems was carried out under their optimum conditions. To demonstrate the use of amperometric and calorimetric biosensor the systems were tested using plasma samples obtained from Songklanagarind Hospital, Prince of Songkla University. The same sample was also analyzed by spectrophotometry using the commercial glucose kit and the hexokinase method. Discrete analysis validation was done with 50 samples. The results of the analyses using the amperometric, calorimetric, the glucose sigma kit and the hexokinase method are shown in Table 34.

Table 34 Glucose concentration in plasma samples obtained by the hexokinase method, glucose Sigma kit and the amperometric and the calorimetric biosensor systems.

Sample	Glucose concentration (mM)				
	Hexokinase method	Glucose Sigma kit	Amperometric system	Calorimetric system	
				GOD+CAT	GOD
1	5.00	5.11	5.17	5.15	5.18
2	19.00	19.45	19.32	19.57	19.39
3	7.89	8.10	7.95	8.29	8.17
4	11.67	12.00	12.10	12.02	12.06
5	6.78	6.95	6.82	7.11	6.98
6	11.00	11.32	11.26	11.28	11.19
7	5.89	6.02	5.95	5.82	5.80
8	6.22	6.47	6.38	6.20	6.18
9	12.22	12.55	12.49	12.63	12.58
10	18.83	19.68	19.50	19.68	19.71
11	11.55	12.36	12.21	12.24	12.27
12	5.00	5.35	5.17	5.52	5.46
13	12.56	12.36	12.40	12.45	12.49
14	5.11	4.84	4.98	4.96	5.00
15	9.11	9.11	9.00	9.02	9.06
16	9.33	9.36	9.47	9.42	9.36
17	5.28	5.47	5.40	5.63	5.58
18	4.61	4.59	4.67	4.62	4.66
19	7.22	7.36	7.46	7.45	7.49
20	14.50	15.87	15.60	15.98	15.89
21	5.55	5.47	5.36	5.39	5.41
22	6.89	6.72	6.88	6.71	6.75
23	9.39	9.49	9.51	9.51	9.55
24	4.55	4.66	4.72	4.61	4.64
25	4.78	4.94	4.85	4.86	4.90
26	8.61	8.55	8.67	8.50	8.57
27	17.00	17.42	17.25	17.35	17.36
28	4.89	4.95	4.97	4.82	4.88
29	8.78	8.67	8.61	8.59	8.58

Table 34 (continues)

Sample	Glucose concentration (mM)				
	Hexokinase method	Glucose Sigma kit	Amperometric system	Calorimetric system	
				GOD+CAT	GOD
30	8.56	8.47	8.45	8.44	8.47
31	4.67	4.82	4.76	4.95	4.88
32	10.00	9.85	9.89	9.92	9.99
33	4.61	4.58	4.50	4.52	4.56
34	8.89	8.92	8.99	9.08	8.91
35	6.11	6.05	6.19	6.07	6.10
36	10.28	10.21	10.37	10.29	10.32
37	10.11	10.25	10.21	10.12	10.18
38	11.27	11.16	11.09	11.07	11.04
39	6.83	6.74	6.72	6.79	6.81
40	4.89	4.93	4.97	4.92	4.86
41	4.89	4.95	5.02	5.09	4.97
42	8.50	8.37	8.43	8.40	8.37
43	8.05	8.11	8.22	8.07	8.10
44	6.94	6.87	6.73	6.83	6.88
45	4.61	4.55	4.47	4.49	4.54
46	5.44	5.32	5.26	5.30	5.37
47	4.55	4.71	4.67	4.59	4.77
48	4.67	4.52	4.59	4.61	4.67
49	7.39	7.25	7.32	7.24	7.20
50	7.56	7.73	7.67	7.70	7.75

3.5 Comparison of the results using amperometric system, glucose Sigma kit and hexokinase method

The amperometric system were validated by comparing the results to those of enzymatic reaction. Comparisons were done for three pairs of different analysis techniques using the regression line method. They are

(I) Amperometric system *Vs* Hexokinase method,

(II) Amperometric system *Vs* Glucose Sigma Kit,

(III) Hexokinase method *Vs* Glucose Sigma Kit.

The results are shown in Figures 46 to 48 and Table 35. In case (II), the calculated slope (m) and the intercept (C) did not differ significantly from the ideal value of 1 and 0 respectively (Table 35), thus these is no evidence for systematic difference between the two method. In contrast the two methods compared in case (I) and (III) differ significant.

To verify the reliability, the two-tailed Wilcoxon signed rank test was also used and the results are shown in Table 36 to 38. In this test the null hypothesis is rejected at a significance level P=0.05 if the experiment value is less than or equal to the tabulate value (Table 2). The Wilcoxon signed rank test (Table 39) confirmed the results obtained by the Regression line method. That is, there is no difference between the amperometric system and the glucose Sigma Kit. However, there are differences between the amperometric and the hexokinase method, and between hexokinase method and the glucose Sigma Kit.

The hexokinase method was done by the autoanalyzer (Hitachi, model 917) at Songklanagarind Hospital. According to its manual, when the hexokinase method was evaluated against a glucose oxidase-oxygen electrode, the latter method would gave, on average, a 3% higher value. This is because the enzyme hexokinase was not only specific to glucose but can also catalyze

mannose, galactose, hexose and fructose. Since the normal concentration of fructose in blood plasma is 6-8 mg/100 ml and this is 7-8% when compare to the normal glucose level (Lehninger,1987). Thus, the catalysis of glucose by hexokinase is interferred by fructose resulting in the difference between the methods.

When the results from the amperometric biosensor system and the glucose Sigma kit were compared to the hexokinase method using the one-tailed Wilcoxon signed rank test (Table 40). It was found that the results of these two methods were significantly higher than the hexokinase method (P=0.05).

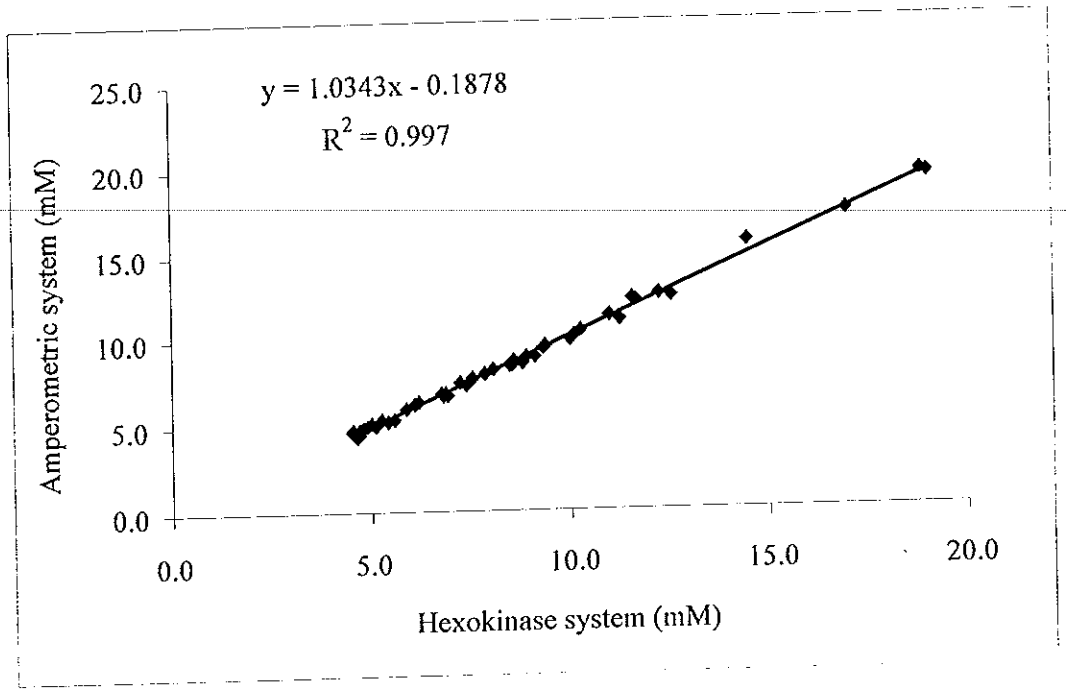


Figure 46 Correlation of the concentration of glucose in plasma sample obtained from the amperometric system and the hexokinase method

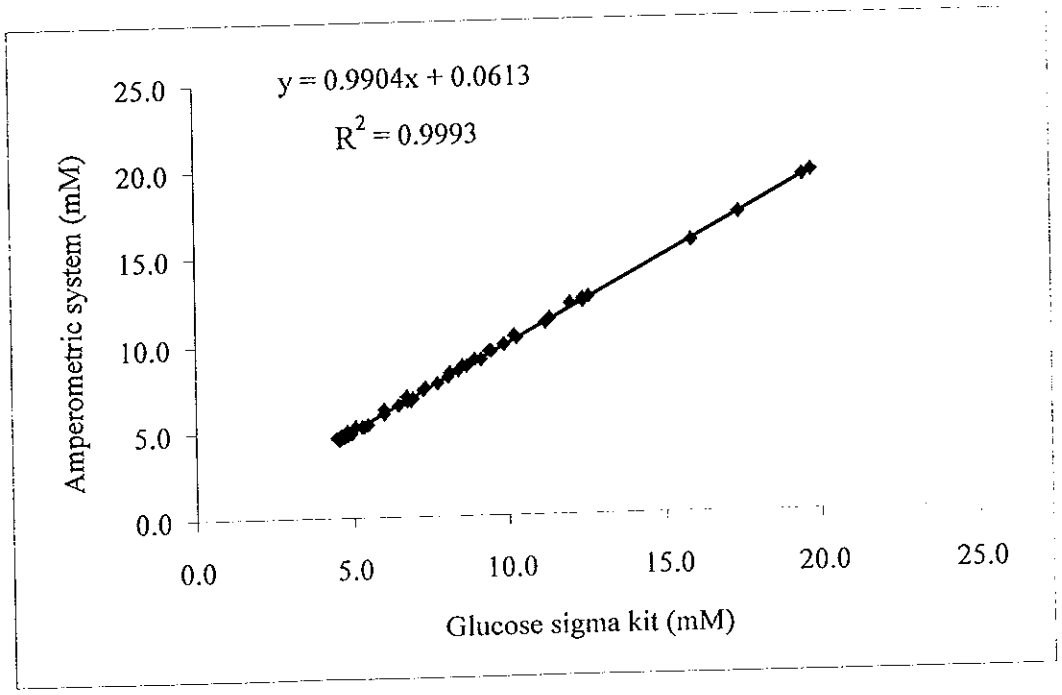


Figure 47 Correlation of the concentration of glucose in plasma sample obtained from the amperometric system and the hexokinase method

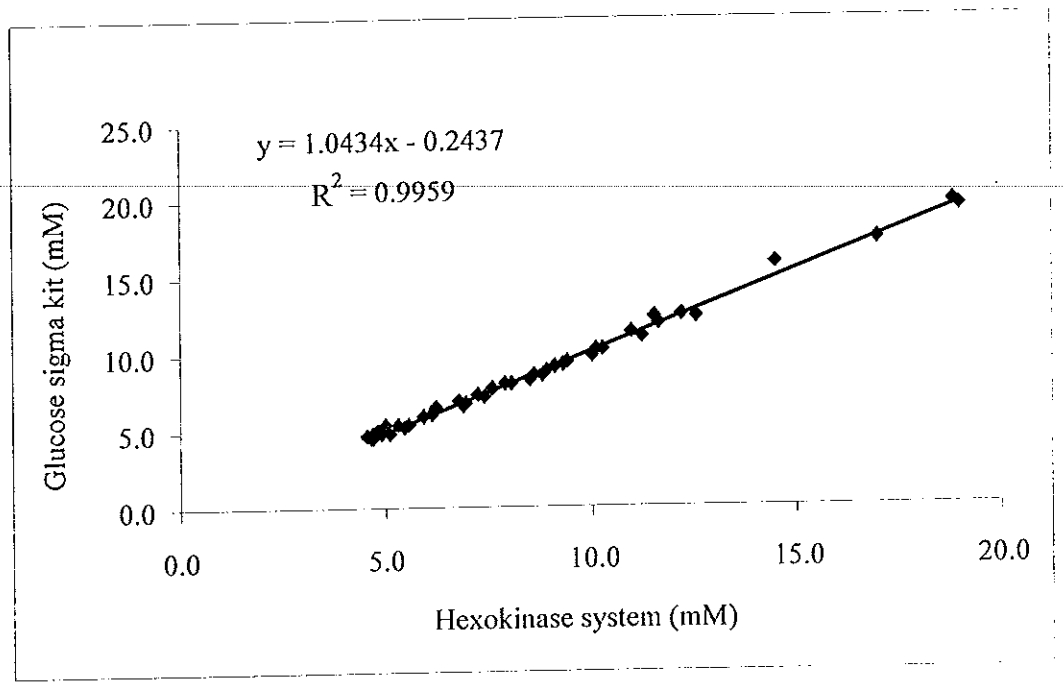


Figure 48 Correlation of the concentration of glucose in plasma sample obtained from the hexokinase method and the glucose Sigma kit

Table 35 Summary of Regression line statistics used in the comparison of the analytical methods

Comparison of two analytical methods	Regression line	R^2	Confidence limits of	
			Slope	Intercept
(I) Amperometric system and Hexokinase method	Y=1.0343x-0.1878	0.9970	1.0343±0.02	-0.187±0.15
(II) Amperometric system and Glucose Sigma Kit	Y=0.9904x+0.0613	0.9993	0.9904±0.01	0.0613±0.07
(III) Hexokinase method and Glucose Sigma Kit	Y=1.0434x-0.2437	0.9959	1.0434±0.02	-0.2437±0.16

Table 36 Application of the Wilcoxon signed rank test for the comparison of the concentration of glucose in plasma samples from the amperometric system and the hexokinase method.

Sample	Glucose concentration (mM) obtained with		The difference of two method (mM)	Rank
	Amperometric system	Hexokinase method		
1	5.17	5.00	0.17	35.0
2	19.32	19.00	0.32	46.0
3	7.95	7.89	0.06	4.5
4	12.10	11.67	0.43	47.0
5	6.82	6.78	0.04	2.0
6	11.26	11.00	0.26	44.0
7	5.95	5.89	0.06	4.5
8	6.38	6.22	0.16	31.5
9	12.49	12.22	0.27	45.0
10	19.50	18.83	0.67	49.0
11	12.21	11.55	0.66	48.0
12	5.17	5.00	0.17	35.0
13	12.40	12.56	-0.16	-31.5
14	4.98	5.11	-0.13	-27.5
15	9.00	9.11	-0.11	-20.5
16	9.47	9.33	0.14	29.5
17	5.40	5.28	0.12	25.0
18	4.67	4.61	0.06	4.5
19	7.46	7.22	0.24	42.0
20	15.60	14.50	1.10	50.0
21	5.36	5.55	-0.19	-40.0
22	6.88	6.89	-0.01	-1.0
23	9.51	9.39	0.12	25.0
24	4.72	4.55	0.17	35.0
25	4.85	4.78	0.07	8.0
26	8.67	8.61	0.06	4.5
27	17.25	17.00	0.25	43.0
28	4.97	4.89	0.08	11.5

Table36 (continues)

Sample	Glucose concentration (mM) obtained with		The difference of two method (mM)	Rank
	Amperometric system	Hexokinase method		
29	8.61	8.78	-0.17	-35.0
30	8.45	8.56	-0.11	-20.5
31	4.76	4.67	0.09	14.5
32	9.89	10.00	-0.11	-20.5
33	4.50	4.61	-0.11	-20.5
34	8.99	8.89	0.10	16.5
35	6.19	6.11	0.08	11.5
36	10.37	10.28	0.09	14.5
37	10.21	10.11	0.10	16.5
38	11.09	11.27	-0.18	-38.5
39	6.72	6.83	-0.11	-20.5
40	4.97	4.89	0.08	11.5
41	5.02	4.89	0.13	27.5
42	8.43	8.50	-0.07	-8.0
43	8.22	8.05	0.17	35.0
44	6.73	6.94	-0.21	-41.0
45	4.47	4.61	-0.14	-29.5
46	5.26	5.44	-0.18	-38.5
47	4.67	4.55	0.12	25.0
48	4.59	4.67	-0.08	-11.5
49	7.32	7.39	-0.07	-8.0
50	7.67	7.56	0.11	20.5
n				50
Sum of positive ranks				862.5
Sum of negative ranks				412.5

Table 37 Application of the Wilcoxon signed rank test for the comparison of the concentration of glucose in plasma samples from the amperometric system and the glucsoe Sigma kit.

Sample	Glucose concentration (mM) obtained with		The difference of two method (mM)	Rank
	Amperometric system	Glucose Sigma kit		
1	5.17	5.11	0.06	14.0
2	19.32	19.45	-0.13	-38.5
3	7.95	8.10	-0.15	-43.5
4	12.10	12.00	0.10	31.5
5	6.82	6.95	-0.13	-38.5
6	11.26	11.32	-0.06	-14.0
7	5.95	6.02	-0.07	-22.0
8	6.38	6.47	-0.09	-29.5
9	12.49	12.55	-0.06	-14.0
10	19.50	19.68	-0.18	-48.5
11	12.21	12.36	-0.15	-43.5
12	5.17	5.35	-0.18	-48.5
13	12.40	12.36	0.04	7.0
14	4.98	4.84	0.14	41.0
15	9.00	9.11	-0.11	-34.5
16	9.47	9.36	0.11	34.5
17	5.40	5.47	-0.07	-22.0
18	4.67	4.59	0.08	27.0
19	7.46	7.36	0.10	31.5
20	15.60	15.87	-0.27	-50.0
21	5.36	5.47	-0.11	-34.5
22	6.88	6.72	0.16	45.5
23	9.51	9.49	0.02	2.5
24	4.72	4.66	0.06	14.0
25	4.85	4.94	-0.09	-29.5
26	8.67	8.55	0.12	37.0
27	17.25	17.42	-0.17	-47.0
28	4.97	4.95	0.02	2.5

Table 37 (continues)

Sample	Glucose concentration (mM) obtained with		The difference of two method (mM)	Rank
	Amperometric system	Glucose Sigma Kit		
29	8.61	8.67	-0.06	-14.0
30	8.45	8.47	-0.02	-2.5
31	4.76	4.82	-0.06	-14.0
32	9.89	9.85	0.04	7.0
33	4.50	4.58	-0.08	-27.0
34	8.99	8.92	0.07	22.0
35	6.19	6.05	0.14	41.0
36	10.37	10.21	0.16	45.5
37	10.21	10.25	-0.04	-7.0
38	11.09	11.16	-0.07	-22.0
39	6.72	6.74	-0.02	-2.5
40	4.97	4.93	0.04	7.0
41	5.02	4.95	0.07	22.0
42	8.43	8.37	0.06	14.0
43	8.22	8.11	0.11	34.5
44	6.73	6.87	-0.14	-41.0
45	4.47	4.55	-0.08	-27.0
46	5.26	5.32	-0.06	-14.0
47	4.67	4.71	-0.04	-7.0
48	4.59	4.52	0.07	22.0
49	7.32	7.25	0.07	22.0
50	7.67	7.73	-0.06	-14.0
n				50
Sum of positive ranks				499.0
Sum of negative ranks				750.0

Table 38 Application of the Wilcoxon signed rank test for the comparison of the concentration of glucose in plasma samples from the glucose Sigma kit system and the hexokinase method.

Sample	Glucose concentration (mM) obtained with		The difference of two method (mM)	Rank
	Glucose Sigma kit	Hexokinase method		
1	5.11	5.00	0.11	19.0
2	19.45	19.00	0.45	46.0
3	8.10	7.89	0.21	38.0
4	12.00	11.67	0.33	42.5
5	6.95	6.78	0.17	34.0
6	11.32	11.00	0.32	41.0
7	6.02	5.89	0.13	23.5
8	6.47	6.22	0.25	39.0
9	12.55	12.22	0.33	42.5
10	19.68	18.83	0.85	48.0
11	12.36	11.55	0.81	47.0
12	5.35	5.00	0.35	44.0
13	12.36	12.56	-0.20	-37.0
14	4.84	5.11	-0.27	-40.0
15	9.11	9.11	0.00	-
16	9.36	9.33	0.03	3.0
17	5.47	5.28	0.19	36.0
18	4.59	4.61	-0.02	-1.0
19	7.36	7.22	0.14	26.0
20	15.87	14.50	1.37	49.0
21	5.47	5.55	-0.08	-14.0
22	6.72	6.89	-0.17	-34.0
23	9.49	9.39	0.10	17.0
24	4.66	4.55	0.11	19.5
25	4.94	4.78	0.16	31.5
26	8.55	8.61	-0.06	-8.5
27	17.42	17.00	0.42	45.0
28	4.95	4.89	0.06	8.5

Table 38 (continues)

Sample	Glucose concentration (mM) obtained with		The difference of two method (mM)	Rank
	Glucose Sigma kit	Hexokinase method		
29	8.67	8.78	-0.11	-19.5
30	8.47	8.56	-0.09	-15.5
31	4.82	4.67	0.15	29.0
32	9.85	10.00	-0.15	-29.0
33	4.58	4.61	-0.03	-3.0
34	8.92	8.89	0.03	3.0
35	6.05	6.11	-0.06	-8.5
36	10.21	10.28	-0.07	-12.5
37	10.25	10.11	0.14	26.0
38	11.16	11.27	-0.11	-19.5
39	6.74	6.83	-0.09	-15.5
40	4.93	4.89	0.04	5.0
41	4.95	4.89	0.06	8.5
42	8.37	8.50	-0.13	-23.5
43	8.11	8.05	0.06	8.5
44	6.87	6.94	-0.07	-12.5
45	4.55	4.61	-0.06	-8.5
46	5.32	5.44	-0.12	-22.0
47	4.71	4.55	0.16	31.5
48	4.52	4.67	-0.15	-29.0
49	7.25	7.39	-0.14	-26.0
50	7.73	7.56	0.17	34.0
n				50
Sum of positive ranks				848.0
Sum of negative ranks				379.0

Table 39 The two-tailed Wilcoxon signed rank test for comparison of two analytical methods. The null hypothesis is rejected if the test statistic T is less than or equal to the critical value (Table 2).

retain null hypothesis = there is no significant difference between the two methods

reject null hypothesis = there is significant difference between the two methods

Comparison of two analytical methods	T = sum of		n	Critical value	Conclusion
	Positive ranks	Negative ranks			
(I) Amperometric system and Hexokinase method	862.5	**412.5**	50	**434**	reject null hypothesis
(II)Amperometric system and Glucose Sigma Kit	**499**	750	50	**434**	retain null hypothesis
(III) Glucose Sigma Kit and Hexokinase method	848	**379**	49	**415**	reject null hypothesis

Table 40 The one-tailed Wilcoxon signed rank test for comparison of two analytical methods. The null hypothesis is rejected if the test statistic T is less than or equal to the critical value (Table 2).

retain null hypothesis = there is no significant difference between the two methods

reject null hypothesis = the first method is significantly higher than the second method

Comparison of two analytical methods	T = sum of		n	Critical value	Conclusion
	Positive ranks	Negative ranks			
Amperometric system and Hexokinase method	862.5	**412.5**	50	**466**	reject null hypothesis
Glucose Sigma Kit and Hexokinase method	848	**379**	49	**446**	reject null hypothesis

3.6 Comparison of the results using the calorimetric system, glucose Sigma kit and the hexokinase method

Discrete analysis validation was done with 50 samples. The results of the analyses using the calorimetric system with two enzyme reactor columns, immobilized glucose oxidase and glucose oxidase co-immobilized with catalase, hexokinase method and glucose Sigma kit. The results are shown in Table 34. Comparisons were done for four pairs of different analysis techniques using the regression line method. They are

(I) Calorimetric system (GOD+CAT) *Vs* Hexokinase method,
(II) Calorimetric system (GOD+CAT) *Vs* Glucose Sigma Kit,
(III) Calorimetric system (GOD) *Vs* Hexokinase method,
(IV) Calorimetric system (GOD) *Vs* Glucose Sigma Kit.

The results are shown in Figures 49 to 52 and Table 41. When compare the results between calorimetric system and the glucose Sigma kit, case (II) and (IV), the calculated slope (m) and the intercept (C) did not differ significantly from the ideal value of 1 and 0 respectively (Table 41), thus these is no evidence for systematic difference between the two methods. In contrast the two methods compared in case (I) and (III) differ significant.

To verify the reliability, the two-tailed Wilcoxon signed rank test was also used and the results are shown in Tables 42 to 45. In this test the null hypothesis is rejected at a significance level P=0.05 if the experiment value is less than or equal to the tabulate value (Table 2). The Wilcoxon signed rank test (Table 46) showed that there was difference between the calorimetric system (GOD) and the hexokinase method but there is as no difference between the calorimetric system (GOD+CAT) and the hexokinase method (Case(I)). This was in contrast to the regression line method. However, when the results

from the calorimetric biosensor system (GOD+CAT) and (GOD) were compared to the hexokinase method using the one-tailed Wilcoxon signed test (Table 47). It was found that the results of these two methods were both higher than the hexokinase method (P=0.05).

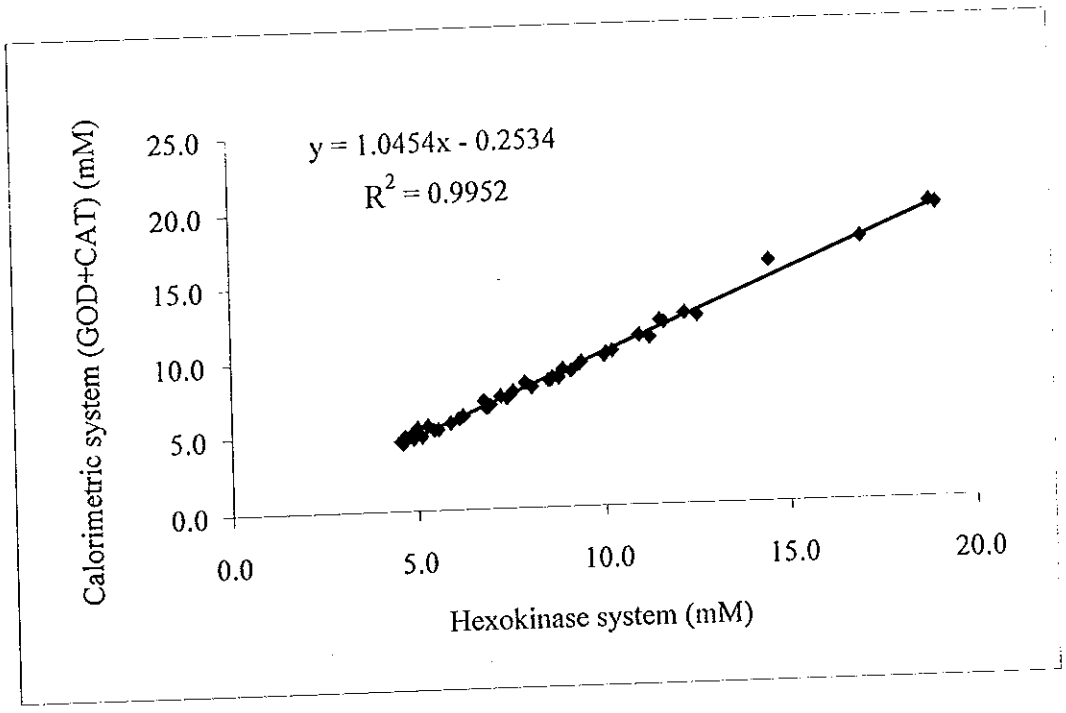


Figure 49 Correlation of the concentration of glucose in plasma sample obtained from the calorimetric system (GOD+CAT) and the hexokinase method

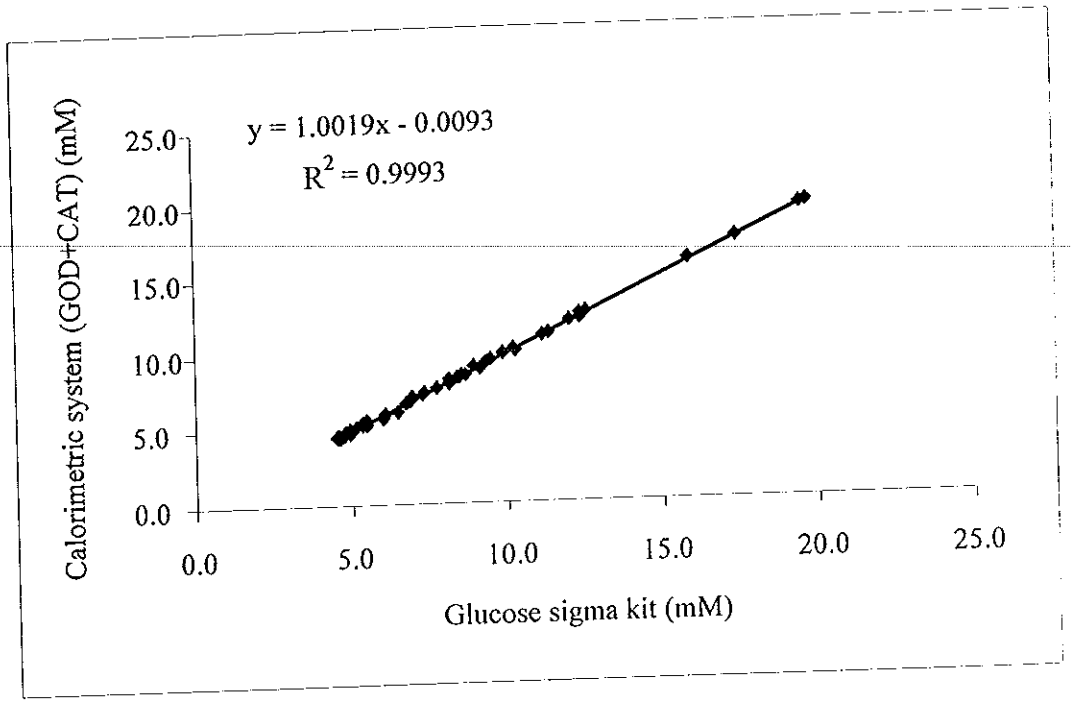


Figure 50 Correlation of the concentration of glucose in plasma sample obtained from the calorimetric system (GOD+CAT) and the glucose Sigma kit

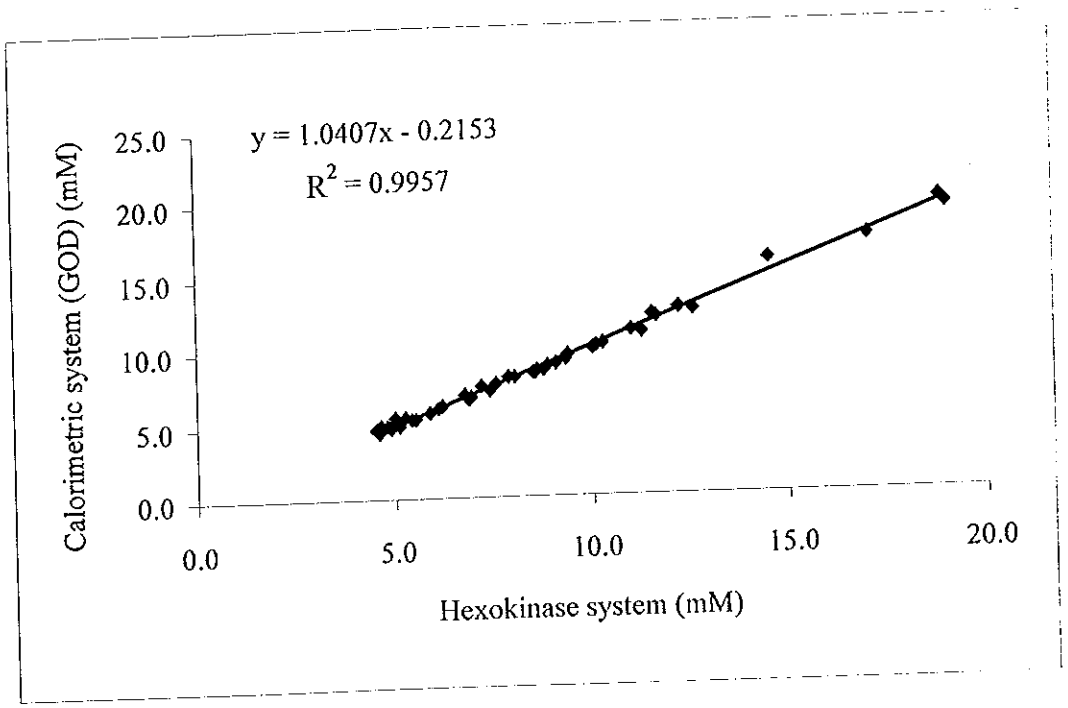


Figure 51 Correlation of the concentration of glucose in plasma sample obtained from the calorimetric system (GOD) and the hexokinase method

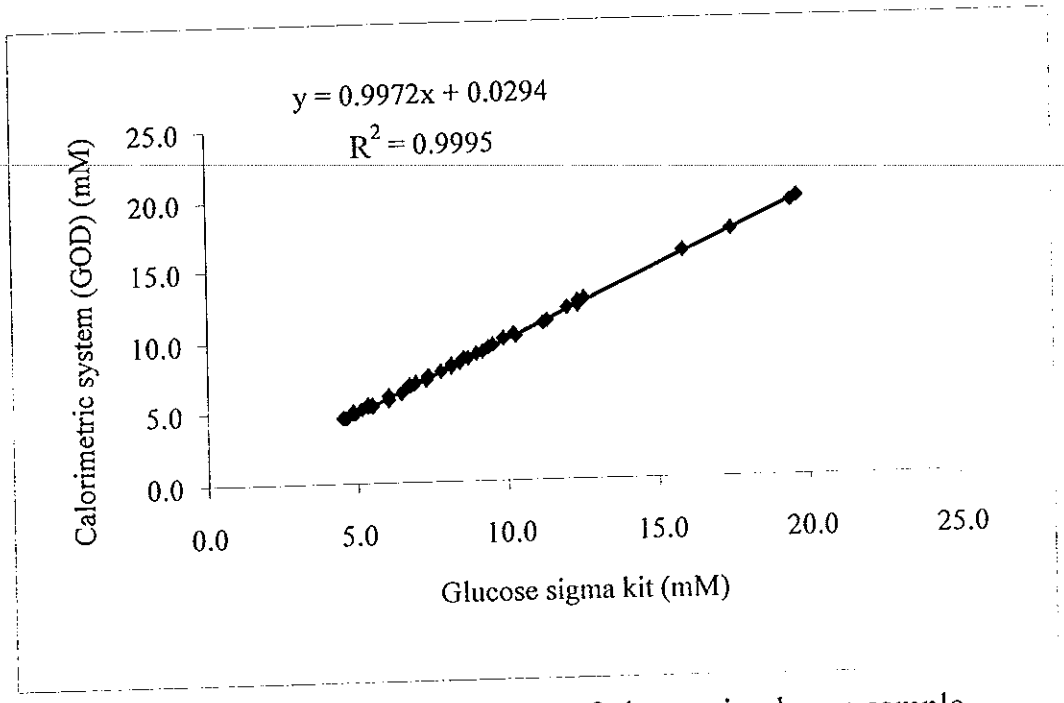


Figure 52 Correlation of the concentration of glucose in plasma sample obtained from the calorimetric system (GOD) and the glucose Sigma kit

Table 41 Summary of Regression line statistics used in the comparison of the analytical methods

Comparison of two analytical methods	Regression line	R^2	Confidence limits of	
			Slope	Intercept
(I) Calorimetric system (GOD+CAT) and Hexokinase method	Y=1.0454x-0.253	0.9952	1.0454±0.02	-0.253±0.19
(II)Calorimetric system (GOD+CAT) and Glucose Sigma Kit	Y=1.0019x-0.0093	0.9993	1.0019±0.01	0.0093±0.07
(III) Calorimetric system (GOD) and Hexokinase method	Y=1.0407x-0.215	0.9957	1.0407±0.02	-0.215±0.18
(IV) Calorimetric system (GOD) and Glucose Sigma Kit	Y=0.9972x+0.0294	0.9995	0.9972±0.01	0.0294±0.06

Table 42 Application of the Wilcoxon signed rank test for the comparison of the concentration of glucose in plasma samples from the calorimetric system (GOD+CAT) and the hexokinase method.

Sample	Glucose concentration (mM) obtained with		The difference of two method (mM)	Rank
	Calorimetric (GOD+CAT)	Hexokinase method		
1	5.15	5.00	0.15	29.0
2	19.57	19.00	0.57	47.0
3	8.29	7.89	0.40	44.0
4	12.02	11.67	0.35	42.0
5	7.11	6.78	0.33	40.0
6	11.28	11.00	0.28	38.5
7	5.82	5.89	-0.07	-12.5
8	6.20	6.22	-0.02	-4.5
9	12.63	12.22	0.41	45.0
10	19.68	18.83	0.85	49.0
11	12.24	11.55	0.69	48.0
12	5.52	5.00	0.52	46.0
13	12.45	12.56	-0.11	-21.0
14	4.96	5.11	-0.15	-29.0
15	9.02	9.11	-0.09	-17.0
16	9.42	9.33	0.09	17.0
17	5.63	5.28	0.35	42.0
18	4.62	4.61	0.01	2.0
19	7.45	7.22	0.23	37.0
20	15.98	14.50	1.48	50.0
21	5.39	5.55	-0.16	-31.0
22	6.71	6.89	-0.18	-32.0
23	9.51	9.39	0.12	24.0
24	4.61	4.55	0.06	10.5
25	4.86	4.78	0.08	14.5
26	8.50	8.61	-0.11	-21.0
27	17.35	17.00	0.35	42.0
28	4.82	4.89	-0.07	-12.5

Table42 (continues)

Sample	Glucose concentration (mM) obtained with		The difference of two method (mM)	Rank
	Calorimetric (GOD+CAT)	Hexokinase method		
29	8.59	8.78	-0.19	-33.5
30	8.44	8.56	-0.12	-24.0
31	4.95	4.67	0.28	38.5
32	9.92	10.00	-0.08	-14.5
33	4.52	4.61	-0.09	-17.0
34	9.08	8.89	0.19	33.5
35	6.07	6.11	-0.04	-8.0
36	10.29	10.28	0.01	2.0
37	10.12	10.11	0.01	2.0
38	11.07	11.27	-0.20	-35.5
39	6.79	6.83	-0.04	-8.0
40	4.92	4.89	0.03	6.0
41	5.09	4.89	0.20	35.5
42	8.40	8.50	-0.10	-19.0
43	8.07	8.05	0.02	4.5
44	6.83	6.94	-0.11	-21.0
45	4.49	4.61	-0.12	-24.0
46	5.30	5.44	-0.14	-26.5
47	4.59	4.55	0.04	8.0
48	4.61	4.67	-0.06	-10.5
49	7.24	7.39	-0.15	-29.0
50	7.70	7.56	0.14	26.5
n				50
Sum of positive ranks				824.0
Sum of negative ranks				451.0

Table 43 Application of the Wilcoxon signed rank test for the comparison of the concentration of glucose in plasma samples from the calorimetric system (GOD+CAT) and the glucose sigma kit.

Sample	Glucose concentration (mM) obtained with		The difference of two method (mM)	Rank
	Calorimetric (GOD+CAT)	Glucose Sigma Kit		
1	5.15	5.11	0.04	13.5
2	19.57	19.45	0.12	36.5
3	8.29	8.10	0.19	47.0
4	12.02	12.00	0.02	5.5
5	7.11	6.95	0.16	44.0
6	11.28	11.32	-0.04	-13.5
7	5.82	6.02	-0.20	-48.0
8	6.20	6.47	-0.27	-49.0
9	12.63	12.55	0.08	26.0
10	19.68	19.68	0.00	-
11	12.24	12.36	-0.12	-36.5
12	5.52	5.35	0.17	46.0
13	12.45	12.36	0.09	31.0
14	4.96	4.84	0.12	36.5
15	9.02	9.11	-0.09	-31.0
16	9.42	9.36	0.06	20.0
17	5.63	5.47	0.16	44.0
18	4.62	4.59	0.03	9.5
19	7.45	7.36	0.09	31.0
20	15.98	15.87	0.11	34.0
21	5.39	5.47	-0.08	-26.0
22	6.71	6.72	-0.01	-2.0
23	9.51	9.49	0.02	5.5
24	4.61	4.66	-0.05	-17.0
25	4.86	4.94	-0.08	-26.0
26	8.50	8.55	-0.05	-17.0
27	17.35	17.42	-0.07	-22.5
28	4.82	4.95	-0.13	-40.0

Table43 (continues)

Sample	Glucose concentration (mM) obtained with		The difference of two method (mM)	Rank
	Calorimetric (GOD+CAT)	Glucose Sigma Kit		
29	8.59	8.67	-0.08	-26.0
30	8.44	8.47	-0.03	-9.5
31	4.95	4.82	0.13	40.0
32	9.92	9.85	0.07	22.5
33	4.52	4.58	-0.06	-20.0
34	9.08	8.92	0.16	44.0
35	6.07	6.05	0.02	5.5
36	10.29	10.21	0.08	26.0
37	10.12	10.25	-0.13	-40.0
38	11.07	11.16	-0.09	-31.0
39	6.79	6.74	0.05	17.0
40	4.92	4.93	-0.01	-2.0
41	5.09	4.95	0.14	42.0
42	8.40	8.37	0.03	9.5
43	8.07	8.11	-0.04	-13.5
44	6.83	6.87	-0.04	-13.5
45	4.49	4.55	-0.06	-20.0
46	5.30	5.32	-0.02	-5.5
47	4.59	4.71	-0.12	-36.5
48	4.61	4.52	0.09	31.0
49	7.24	7.25	-0.01	-2.0
50	7.70	7.73	-0.03	-9.5
n				49
Sum of positive ranks				482.5
Sum of negative ranks				740.5

Table 44 Application of the Wilcoxon signed rank test for the comparison of the concentration of glucose in plasma samples from the calorimetric system (GOD) and the hexokinase method.

Sample	Glucose concentration (mM) obtained with		The difference of two method (mM)	Rank
	Calorimetric (GOD)	Hexokinase method		
1	5.18	5.00	0.18	30.0
2	19.39	19.00	0.39	44.5
3	8.17	7.89	0.28	40.0
4	12.06	11.67	0.39	44.5
5	6.98	6.78	0.20	34.5
6	11.19	11.00	0.19	32.0
7	5.80	5.89	-0.09	-22.0
8	6.18	6.22	-0.04	-9.0
9	12.58	12.22	0.36	42.5
10	19.71	18.83	0.88	48.0
11	12.27	11.55	0.72	47.0
12	5.46	5.00	0.46	46.0
13	12.49	12.56	-0.07	-17.5
14	5.00	5.11	-0.11	-24.0
15	9.06	9.11	-0.05	-12.5
16	9.36	9.33	0.03	6.5
17	5.58	5.28	0.30	41.0
18	4.66	4.61	0.05	12.5
19	7.49	7.22	0.27	39.0
20	15.89	14.50	1.39	49.0
21	5.41	5.55	-0.14	-27.5
22	6.75	6.89	-0.14	-27.5
23	9.55	9.39	0.16	29.0
24	4.64	4.55	0.09	22.0
25	4.90	4.78	0.12	25.0
26	8.57	8.61	-0.04	-9.0
27	17.36	17.00	0.36	42.5
28	4.88	4.89	-0.01	-2.0

Table44 (continues)

Sample	Glucose concentration (mM) obtained with		The difference of two method (mM)	Rank
	Calorimetric (GOD)	Hexokinase method		
29	8.58	8.78	-0.20	-34.5
30	8.47	8.56	-0.09	-22.0
31	4.88	4.67	0.21	36.0
32	9.99	10.00	-0.01	-2.0
33	4.56	4.61	-0.05	-12.5
34	8.91	8.89	0.02	4.5
35	6.10	6.11	-0.01	-2.0
36	10.32	10.28	0.04	9.0
37	10.18	10.11	0.07	17.5
38	11.04	11.27	-0.23	-38.0
39	6.81	6.83	-0.02	-4.5
40	4.86	4.89	-0.03	-6.5
41	4.97	4.89	0.08	20.0
42	8.37	8.50	-0.13	-26.0
43	8.10	8.05	0.05	12.5
44	6.88	6.94	-0.06	-15.0
45	4.54	4.61	-0.07	-17.5
46	5.37	5.44	-0.07	-17.5
47	4.77	4.55	0.22	37.0
48	4.67	4.67	0.00	-
49	7.20	7.39	-0.19	-32.0
50	7.75	7.56	0.19	32.0
n				49
Sum of positive ranks				826.5
Sum of negative ranks				371.0

Table 45 Application of the Wilcoxon signed rank test for the comparison of the concentration of glucose in plasma samples from the calorimetric system (GOD) and the glucose sigma kit.

Sample	Glucose concentration (mM) obtained with		The difference of two method (mM)	Rank
	Calorimetric (GOD)	Glucose Sigma Kit		
1	5.18	5.11	0.07	30.0
2	19.39	19.45	-0.06	-23.0
3	8.17	8.10	0.07	30.0
4	12.06	12.00	0.06	23.0
5	6.98	6.95	0.03	12.5
6	11.19	11.32	-0.13	41.0
7	5.80	6.02	-0.22	-46.0
8	6.18	6.47	-0.29	-47.0
9	12.58	12.55	0.03	12.5
10	19.71	19.68	0.03	12.5
11	12.27	12.36	-0.09	-34.5
12	5.46	5.35	0.11	37.0
13	12.49	12.36	0.13	41.0
14	5.00	4.84	0.16	45.0
15	9.06	9.11	-0.05	-17.5
16	9.36	9.36	0.00	-
17	5.58	5.47	0.11	37.0
18	4.66	4.59	0.07	30.0
19	7.49	7.36	0.13	41.0
20	15.89	15.87	0.02	7.5
21	5.41	5.47	-0.06	-23.0
22	6.75	6.72	0.03	12.5
23	9.55	9.49	0.06	23.0
24	4.64	4.66	-0.02	-7.5
25	4.90	4.94	-0.04	-15.0
26	8.57	8.55	0.02	7.5
27	17.36	17.42	-0.06	-23.0
28	4.88	4.95	-0.07	-30.0

Table45 (continues)

Sample	Glucose concentration (mM) obtained with		The difference of two method (mM)	Rank
	Calorimetric (GOD)	Glucose Sigma Kit		
29	8.58	8.67	-0.09	-34.5
30	8.47	8.47	0.00	-
31	4.88	4.82	0.06	23.0
32	9.99	9.85	0.14	43.0
33	4.56	4.58	-0.02	-7.5
34	8.91	8.92	-0.01	-2.5
35	6.10	6.05	0.05	17.5
36	10.32	10.21	0.11	37.0
37	10.18	10.25	-0.07	-30.0
38	11.04	11.16	-0.12	-39.0
39	6.81	6.74	0.07	30.0
40	4.86	4.93	-0.07	-30.0
41	4.97	4.95	0.02	7.5
42	8.37	8.37	0.00	-
43	8.10	8.11	-0.01	-2.5
44	6.88	6.87	0.01	2.5
45	4.54	4.55	-0.01	-2.5
46	5.37	5.32	0.05	17.5
47	4.77	4.71	0.06	23.0
48	4.67	4.52	0.15	44.0
49	7.20	7.25	-0.05	-17.5
50	7.75	7.73	0.02	7.5
n				47
Sum of positive ranks				654.5
Sum of negative ranks				473.5

Table 46 The two-tailed Wilcoxon signed rank test for comparison of two analytical methods. The null hypothesis is rejected if the test statistic T is less than or equal to the critical value (Table 2).

retain null hypothesis = there is no significant difference between the two methods

reject null hypothesis = there is significant difference between the two methods

Comparison of two analytical methods	T = sum of		n	Critical value	Conclusion
	Positive ranks	Negative ranks			
Calorimetric system (GOD+CAT) and Hexokinase method	824.0	**451.0**	50	**434**	retain null hypothesis
Calorimetric system (GOD+CAT) and Glucose Sigma Kit	**482.5**	740.5	49	**415**	retain null hypothesis
Calorimetric system (GOD) and Hexokinase method	826.5	**371.0**	49	**415**	reject null hypothesis
Calorimetric system (GOD) and Glucose Sigma Kit	654.5	**473.5**	47	**379**	retain null hypothesis

Table 47 The one-tailed Wilcoxon signed rank test for comparison of two analytical methods. The null hypothesis is rejected if the test statistic T is less than or equal to the critical value (Table 2).

retain null hypothesis = there is no significant difference between the two methods

reject null hypothesis = the first method is significantly higher than the second method

Comparison of two analytical methods	T = sum of		n	Critical value	Conclusion
	Positive ranks	Negative ranks			
Calorimetric system (GOD+CAT)and Hexokinase method	824.0	**451.0**	50	**466**	reject null hypothesis
Calorimetric system (GOD) and Hexokinase method	826.5	**371.0**	49	**446**	reject null hypothesis

3.7 Comparison of the results using amperometric and calorimetric biosensor system

Discrete analysis validation was done with 50 samples. The results of analyses using the amperometric biosensor system and the calorimeric biosensor system with two enzyme reactor columns, immobilized glucose oxidase and glucose oxidase co-immobilized with catalase. Comparisons were done for three pairs of different analysis techniques using the regression line method. They are

(I) Amperometric system *Vs* Calorimetric system (GOD+CAT),

(II) Amperometric system *Vs* Calorimetric system (GOD),

(III) Calorimetric system (GOD+CAT) *Vs* Calorimetric system (GOD)

The results are shown in Figures 53 to 55 and Table 48. In all cases, the calculated slope (m) and the intercept (C) did not differ significantly from the ideal value of 1 and 0 respectively (Table 48). Thus these is no evidence for systematic difference among each methods.

To verify the reliability, the two-tailed Wilcoxon signed rank test was also used and the results are shown in Tables 49 to 51. In this test the null hypothesis is rejected at a significance level P=0.05 if the experiment value is less than or equal to the tabulate value (Table 2). In all three cases, the null hypothesis are retained (Table 51). That is there is no evidence for systematic difference between the result of the amperometric and the calorimetric biosensor system.

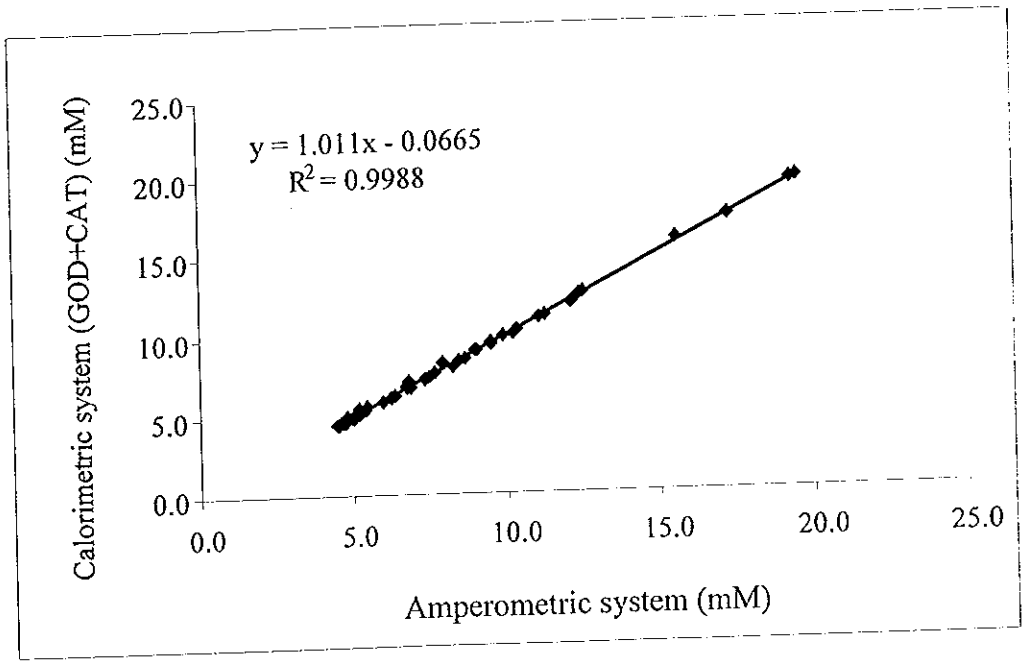


Figure 53 Correlation of the concentration of glucose in plasma sample obtained from the amperometric system and the calorimetric system (GOD+CAT)

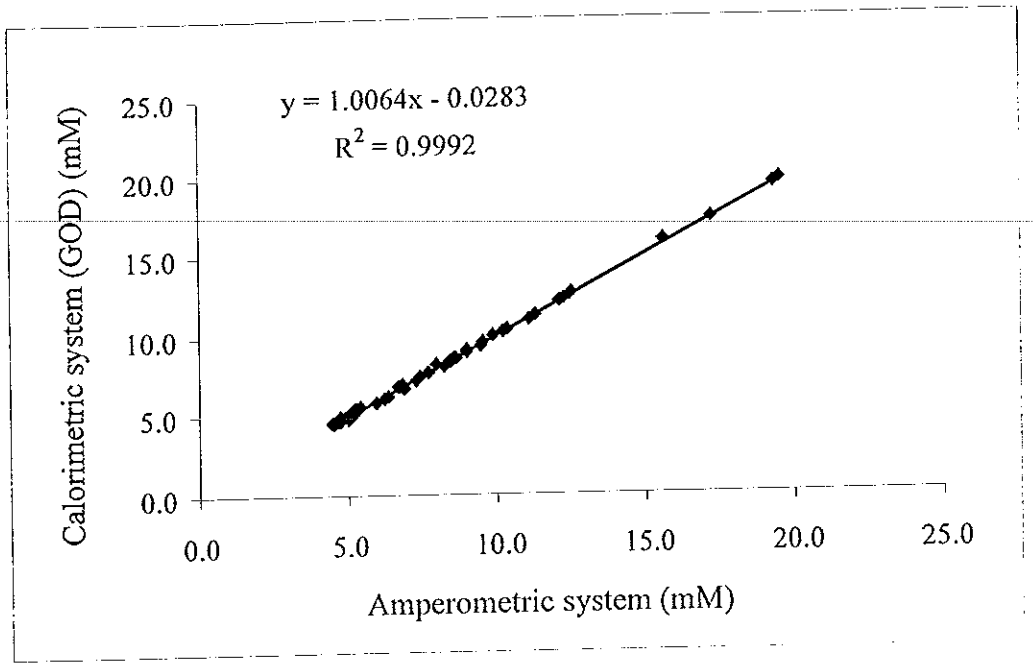


Figure 54 Correlation of the concentration of glucose in plasma sample obtained from the amperometric system and the calorimetric system (GOD)

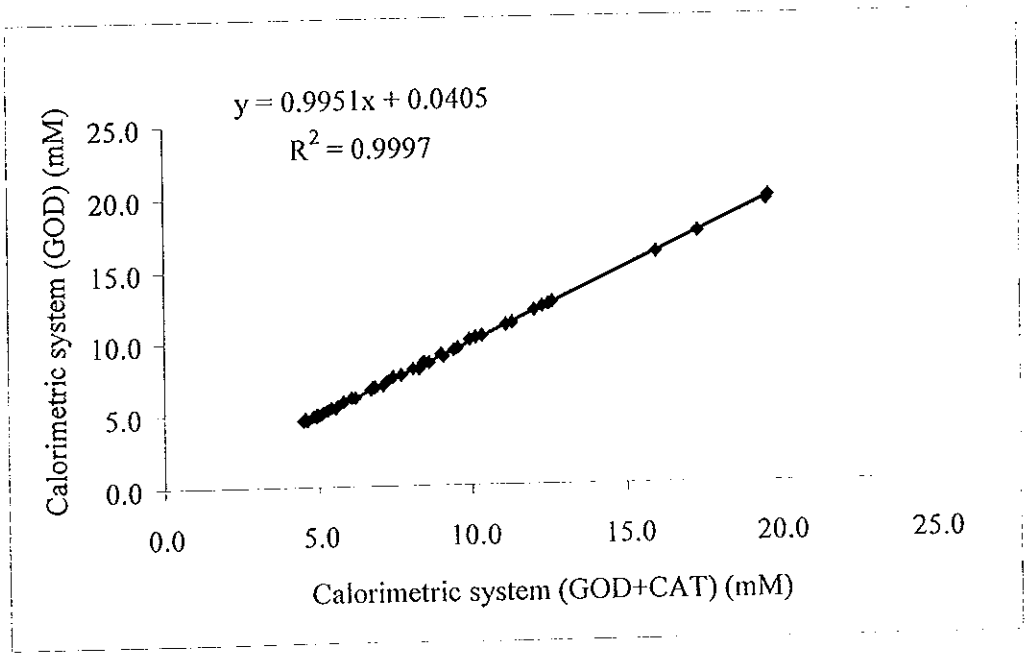


Figure 55 Correlation of the concentration of glucose in plasma sample obtained from the calorimetric system (GOD+CAT) and the calorimetric system (GOD)

Table 48 Summary of Regression line statistics used in the comparison of the analytical methods

Comparison of two analytical methods	Regression line	R^2	Confidence limits of	
			Slope	Intercept
Amperometric system and Calorimetric system (GOD+CAT)	Y=1.011x-0.0665	0.9988	1.001±0.01	-0.0665±0.09
Amperometric system and Calorimetric system (GOD)	Y=1.0064x-0.0283	0.9992	1.0064±0.01	-0.0283±0.08
Calorimetric system (GOD+CAT) and Calorimetric system (GOD)	Y=0.9951x+0.0405	0.9997	0.9951±0.01	0.0405±0.05

Table 49 Application of the Wilcoxon signed rank test for the comparison of the concentration of glucose in plasma samples from the amperometric system and Calorimetric system (GOD+CAT)

Sample	Glucose concentration (mM) obtained with		The difference of two method (mM)	Rank
	Amperometric	Calorimetric(GOD+CAT)		
1	5.17	5.15	0.02	8.0
2	19.32	19.57	-0.25	-45.0
3	7.95	8.29	-0.34	-47.0
4	12.10	12.02	0.08	26.5
5	6.82	7.11	-0.29	-46.0
6	11.26	11.28	-0.02	-8.0
7	5.95	5.82	0.13	35.0
8	6.38	6.20	0.18	41.5
9	12.49	12.63	-0.14	-36.0
10	19.50	19.68	-0.18	-41.5
11	12.21	12.24	-0.03	-15.0
12	5.17	5.52	-0.35	-48.0
13	12.40	12.45	-0.05	-20.5
14	4.98	4.96	0.02	8.0
15	9.00	9.02	-0.02	-8.0
16	9.47	9.42	0.05	20.5
17	5.40	5.63	-0.23	-44.0
18	4.67	4.62	0.05	20.5
19	7.46	7.45	0.01	2.0
20	15.60	15.98	-0.38	-49.0
21	5.36	5.39	-0.03	-15.0
22	6.88	6.71	0.17	39.5
23	9.51	9.51	0.00	-
24	4.72	4.61	0.11	33.0
25	4.85	4.86	-0.01	-2.0
26	8.67	8.50	0.17	39.5
27	17.25	17.35	-0.10	-31.5
28	4.97	4.82	0.15	37.5

Table49 (continues)

Sample	Glucose concentration (mM) obtained with		The difference of two method (mM)	Rank
	Amperometric	Calorimetric (GOD+CAT)		
29	8.61	8.59	0.02	8.0
30	8.45	8.44	0.01	2.0
31	4.76	4.95	-0.19	-43.0
32	9.89	9.92	-0.03	-15.0
33	4.50	4.52	-0.02	-8.0
34	8.99	9.08	-0.09	-29.5
35	6.19	6.07	0.12	34.0
36	10.37	10.29	0.08	26.5
37	10.21	10.12	0.09	29.5
38	11.09	11.07	0.02	8.0
39	6.72	6.79	-0.07	-23.5
40	4.97	4.92	0.05	20.5
41	5.02	5.09	-0.07	-23.5
42	8.43	8.40	0.03	15.0
43	8.22	8.07	0.15	37.5
44	6.73	6.83	-0.10	-31.5
45	4.47	4.49	-0.02	-8.0
46	5.26	5.30	-0.04	-18.0
47	4.67	4.59	0.08	26.5
48	4.59	4.61	-0.02	-8.0
49	7.32	7.24	0.08	26.5
50	7.67	7.70	-0.03	-15.0
n				49
Sum of positive ranks				545.5
Sum of negative ranks				679.5

Table 50 Application of the Wilcoxon signed rank test for the comparison of the concentration of glucose in plasma samples from the amperometric system and calorimetric system (GOD)

Sample	Glucose concentration (mM) obtained with		The difference of two method (mM)	Rank
	Amperometric	Calorimetric(GOD)		
1	5.17	5.18	-0.01	-1.5
2	19.32	19.39	-0.07	-20.0
3	7.95	8.17	-0.22	-48.0
4	12.10	12.06	0.04	8.5
5	6.82	6.98	-0.16	-44.0
6	11.26	11.19	0.07	20.0
7	5.95	5.80	0.15	42.5
8	6.38	6.18	0.20	46.0
9	12.49	12.58	-0.09	-28.0
10	19.50	19.71	-0.21	-47.0
11	12.21	12.27	-0.06	-16.5
12	5.17	5.46	-0.29	-50.0
13	12.40	12.49	-0.09	-28.0
14	4.98	5.00	-0.02	-3.5
15	9.00	9.06	-0.06	-16.5
16	9.47	9.36	0.11	35.5
17	5.40	5.58	-0.18	-45.0
18	4.67	4.66	0.01	1.5
19	7.46	7.49	-0.03	-6.0
20	15.60	15.89	-0.29	-49.0
21	5.36	5.41	-0.05	-12.0
22	6.88	6.75	0.13	41.0
23	9.51	9.55	-0.04	-8.5
24	4.72	4.64	0.08	23.5
25	4.85	4.90	-0.05	-12.0
26	8.67	8.57	0.10	32.0
27	17.25	17.36	-0.11	-35.5
28	4.97	4.88	0.09	28.0

Table50 (continues)

Sample	Glucose concentration (mM) obtained with		The difference of two method (mM)	Rank
	Amperometric	Calorimetric (GOD)		
29	8.61	8.58	0.03	6.0
30	8.45	8.47	-0.02	-3.5
31	4.76	4.88	-0.12	-39.0
32	9.89	9.99	-0.10	-32.0
33	4.50	4.56	-0.06	-16.5
34	8.99	8.91	0.08	23.5
35	6.19	6.10	0.09	28.0
36	10.37	10.32	0.05	12.0
37	10.21	10.18	0.03	6.0
38	11.09	11.04	0.05	12.0
39	6.72	6.81	-0.09	-28.0
40	4.97	4.86	0.11	35.5
41	5.02	4.97	0.05	12.0
42	8.43	8.37	0.06	16.5
43	8.22	8.10	0.12	39.0
44	6.73	6.88	-0.15	-42.5
45	4.47	4.54	-0.07	-20.0
46	5.26	5.37	-0.11	-35.5
47	4.67	4.77	-0.10	-32.0
48	4.59	4.67	-0.08	-23.5
49	7.32	7.20	0.12	39.0
50	7.67	7.75	-0.08	-23.5
n				50
Sum of positive ranks				508.0
Sum of negative ranks				758.5

Table 51 Application of the Wilcoxon signed rank test for the comparison of the concentration of glucose in plasma samples from the calorimetric system (GOD+CAT) and calorimetric system (GOD)

Sample	Glucose concentration (mM) obtained with		The difference of two method (mM)	Rank
	Calorimetric(GOD+CAT)	Calorimetric(GOD)		
1	5.15	5.18	-0.03	-11.5
2	19.57	19.39	0.18	-49.0
3	8.29	8.17	0.12	45.5
4	12.02	12.06	-0.04	-22.0
5	7.11	6.98	0.13	47.0
6	11.28	11.19	0.09	43.5
7	5.82	5.80	0.02	4.5
8	6.20	6.18	0.02	4.5
9	12.63	12.58	0.05	30.0
10	19.68	19.71	-0.03	-11.5
11	12.24	12.27	-0.03	-11.5
12	5.52	5.46	0.06	-35.5
13	12.45	12.49	-0.04	-22.0
14	4.96	5.00	-0.04	-22.0
15	9.02	9.06	-0.04	-22.0
16	9.42	9.36	0.06	35.5
17	5.63	5.58	0.05	30.0
18	4.62	4.66	-0.04	-22.0
19	7.45	7.49	-0.04	-22.0
20	15.98	15.89	0.09	43.5
21	5.39	5.41	-0.02	-4.5
22	6.71	6.75	-0.04	-22.0
23	9.51	9.55	-0.04	-22..0
24	4.61	4.64	-0.03	-11.5
25	4.86	4.90	-0.04	-22.0
26	8.50	8.57	-0.07	-40.5
27	17.35	17.36	-0.01	-1.5
28	4.82	4.88	-0.06	-35.5

Table51 (continues)

Sample	Glucose concentration (mM) obtained with		The difference of two method (mM)	Rank
	Calorimetric(GOD+CAT)	Calorimetric (GOD)		
29	8.59	8.58	0.01	1.5
30	8.44	8.47	-0.03	-11.5
31	4.95	4.88	0.07	40.5
32	9.92	9.99	-0.07	-40.5
33	4.52	4.56	-0.04	-22.0
34	9.08	8.91	0.17	48.0
35	6.07	6.10	-0.03	-11.5
36	10.29	10.32	-0.03	-11.5
37	10.12	10.18	-0.06	-35.5
38	11.07	11.04	0.03	11.5
39	6.79	6.81	-0.02	-4.5
40	4.92	4.86	0.06	35.5
41	5.09	4.97	0.12	45.5
42	8.40	8.37	0.03	11.5
43	8.07	8.10	-0.03	-11.5
44	6.83	6.88	-0.05	-30.0
45	4.49	4.54	-0.05	-30.0
46	5.30	5.37	-0.07	-40.5
47	4.59	4.77	-0.18	-49.0
48	4.61	4.67	-0.06	-35.5
49	7.24	7.20	0.04	22.0
50	7.70	7.75	-0.05	-30.0
n				50
Sum of positive ranks				585.5
Sum of negative ranks				689.5

Table 52 The two-tailed Wilcoxon signed rank test for comparison of two analytical methods. The null hypothesis is rejected if the test statistic T is less than or equal to the critical value (Table 2).

retain null hypothesis = there is no significant difference between the two methods

reject null hypothesis = there is significant difference between the two methods

Comparison of two analytical methods	T = sum of		n	Critical value	Conclusion
	Positive ranks	Negative ranks			
Amperometric system and Calorimetric system (GOD+CAT)	**545.5**	679.5	49	**415**	retain null hypothesis
Amperometric system and Calorimetric system (GOD)	**508.0**	758.5	50	**434**	retain null hypothesis
Calorimetric system (GOD+CAT) and Calorimetric system (GOD)	**585.5**	689.5	50	**434**	retain null hypothesis

Chapter 4

Conclusions

The efficiency of two transducers, oxygen electrode and thermistor, were compared in the biosensor systems to determine glucose concentration in plasma samples. In both biosensors, the evaluation was done in the enzyme reactor column flow injection system. The immobilization of enzyme glucose oxidase was done by covalent binding on silica gel. An oxygen electrode or a thermistor was used to detect the change resulting from the oxidation reaction between the enzyme glucose oxidase and its substrate, glucose.

In the amperometric system, an oxygen electrode was used to detect the change in oxygen. In the calorimetric system the thermistor was used to detect the heat change. Both systems used a column with immobilized glucose oxidase to catalyse the oxidation reaction of glucose. In addition a column contained glucose oxidase co-immobilized with catalase was also tested in the calorimetric system. Both systems used a dialyser to filter off large molecules, thus, preventing them from blocking the enzyme reactor. Parameters influencing the performance of the systems were studied and optimized to be used for determination of glucose in plasma samples. The optimum condition of these systems were

	amperometric system	calorimetric system (both enzyme reactor columns)
flow rate of buffer line	0.75 ml min^{-1}	0.75 ml min^{-1}
flow rate of sample line	0.30 ml min^{-1}	0.40 ml min^{-1}
buffer	0.1 M phosphate pH 7.20	0.1 M phosphate pH 7.00
sample volume	500 µl	500 µl

To detect glucose in real plasma samples 0.9% NaCl was added to the buffer used in the sample line and to standard glucose solution since it would provide an isotonic condition for the plasma samples.

For the amperometric system, the linear range and the analysis time was 0.1-6 mM and 8-10 min, respectively. The stability of immobilized glucose oxidase on silica gel has been investigated intermittently over a period of 5 months. After 312 h operation time the enzyme reactor column retained about 67% of its original sensitivity.

In the calorimetric system the linear range of the enzyme reactor columns with glucose oxidase co-immobilized catalase and immobilized glucose oxidase were 0.1-8.0 mM and 0.3-8.0 mM, respectively. Both enzyme reactor columns had the same analysis time, 8-9 min. After 203 h the activity of the reactor with two enzyme decreased about 19% and decrease 12% in the column with glucose oxidase.

To test the biosensor systems, the same plasma samples were analysed by the biosensor, the autoanalyzer (Hitachi, 917) by Songklanagarind Hospital (Hexokinase method), and the commercial glucose Sigma kit. The results were compared using the regression line method and the two-tailed Wilcoxon signed rank test. The results indicated good agreement between the biosensor systems and the commercial glucose Sigma kit. But when compared the biosensor systems or the glucose Sigma kit to the hexokinase method they differred significantly. When the results were further tested by the one-tailed Wilcoxon

singed rank test, the test statistic indicated that the biosensor systems and the glucose Sigma kit gave significantly higher analysis results than the hexokinase method. This is agreed well with the hexokinase method manual (Manual of Gluco-quant®Glucose/HK, 1999) which indicated that when the hexokinase method was evaluated against a glucose oxidase-oxygen electrode, the latter method would gave, on average, a 3% higher value. This is because the enzyme hexokinase was not only specific to glucose but can also catalyze other sugars in the human plasma.

The advantages of the proposed method are that it does not require such a sophisticated equipment like an autoanalyzer or the elimination of proteins and coloration as required for the conventional spectrophotometric method or does it require any other pretreatment.

For the calorimetric biosensor system, it was found that the enzyme reactor column that contained two enzymes, glucose oxidase co-immobilized with catalase, had higher sensitivities than the enzyme reactor column with only glucose oxidase (58.8%). This was due to the addition of heat caused by the catalysis reaction of hydrogen peroxide by catalase. However, both enzyme reactor columns had the same analysis time and the detection limit of both systems were sufficient to analyse glucose in plasma samples.

When compared the amperometric and the calorimetric systems, the analysis of glucose concentration in plasma samples indicated good agreement between these two biosensors. The amperometric system gave a slightly higher response than the calorimetric system, but its analysis time was longer. The temperature control for the oxygen electrode was also simpler. However, the calorimetric system can be used to detect wider range of reaction, *i.e.*, all reactions which generated or consumed heat. In addition there would be less interference from other electrochemical reaction than the amperometric method.

In conclusion, the biosensor systems are reliable sensors which can be used to determine glucose in human plasma. The preparation of the enzyme reactor is relatively simple and its good response stability together with simple buffer reagent make it more economical than the automated and spectrophotometric systems.

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