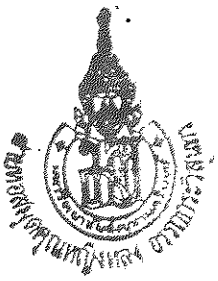
The Determination of Urea Using Enzyme Sensor

Woraphot Saelim

Master of Science Thesis in Analytical Chemistry

Prince of Songkla University

2000



Thesis Title	The Determination of Urea Using Enzyme Sensor
Author	Mr. Woraphot Saelim
Major Program	Analytical Chemistry

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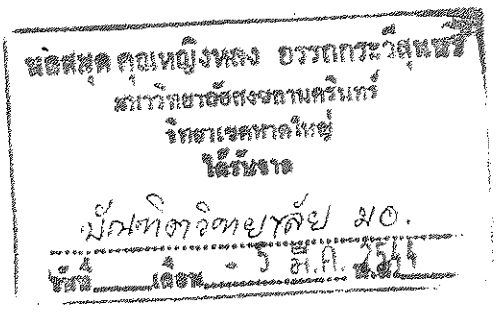
Dean, Graduate School



ชื่อวิทยานิพนธ์ การหาปริมาณยูเรียโดยเอนไซม์เซนเซอร์

ผู้เขียน นายวรพจน์ แซ่ลิ่ม

สาขาวิชา เคมีวิเคราะห์

ปีการศึกษา 2543

บทคัดย่อ

เอนไซม์เซนเซอร์สำหรับยูเรียใช้เอนไซม์ยูริเอสที่ตรึงอยู่บนเม็ดแก้วที่มีรูพรุน (porous glass) เร่งปฏิกิริยาไฮโดรไลซีสของยูเรียได้ผลผลิตที่มีประจุซึ่งทำให้สภาพนำไฟฟ้าของสารละลายเพิ่มขึ้น และตรวจวัดการเปลี่ยนแปลงของสภาพนำไฟฟ้าโดยคอนดักติวิตีอิเล็กโทรด (conductivity electrode) ระบบที่ใช้ในงานวิจัยนี้ใช้ไดอะไลเซอร์ (dialyser) ช่วยป้องกันสารโมเลกุลใหญ่ไม่ให้เข้าไปถึงเอนไซม์รีแอกเตอร์ เนื่องจากจะทำให้เกิดการอุดตันของระบบ โดยสารตัวอย่างจะถูกผ่านเป็นช่วงๆในสารละลายบัฟเฟอร์ที่ไหลอย่างต่อเนื่อง โมเลกุลของยูเรียจากสารตัวอย่างจะผ่านไดอะไลซิส เมมเบรน เข้าไปในบัฟเฟอร์ที่ไหลผ่านอีกด้านหนึ่งของเมมเบรน และจะถูกผ่านต่อไปยังเอนไซม์รีแอกเตอร์ที่บรรจุเอมไซม์สภาวะตรึง สภาพนำไฟฟ้าที่เพิ่มขึ้นซึ่งมีความสัมพันธ์กับความเข้มข้นของยูเรียในสารละลายจะถูกตรวจวัดและบันทึกไว้

การศึกษาเปรียบเทียบระหว่างระบบไหลผ่านกับระบบโฟลอินเจกชัน และไดอะไลเซอร์ที่มีพื้นที่ต่างกัน พบว่าระบบที่เหมาะสมที่สุดคือ ระบบโฟลอินเจกชัน ที่ใช้ไดอะไลเซอร์ตัวใหญ่ (พื้นที่ในการแพร่ 1.5 x 298 ตารางมิลลิเมตร) โดยให้ความสัมพันธ์เชิงเส้น (linear range) ในช่วง 0.5-10 มิลลิโมลาร์ (r^2 = 0.9955) เวลาที่ใช้ในการวิเคราะห์ประมาณ 25 นาที และอายุการใช้งานของเอนไซม์รีแอกเตอร์มากกว่า 260 ชั่วโมง (หรือ ใช้เป็นช่วงๆได้มากกว่า 6 เดือน)

ระบบนี้เมื่อใช้วิเคราะห์หาปริมาณยูเรียในตัวอย่างซีรัม (serum) และเปรียบเทียบผลกับวิธีมาตรฐานสองวิธี (Berthelot และ Fearon reactions) โดยใช้ผลทางสถิติของวิธี Regression line และ Wilcoxon signed rank test พบว่าผลการทดลองของทั้งสามวิธีสอดคล้องกัน

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Academic Year	2000

Abstract

An enzyme-based sensor for the determination of urea is described. In this system urease was immobilized to porous glass and conductivity electrodes were used to measure the increase in conductivity of the sample solution due to the hydrolysis of urea into charged products. The system used a dialyser to filter off large molecules, thus, preventing them from blocking the enzyme reactor. The sample solutions were introduced as pulses in a continuous flow of buffer. Urea molecules from the sample passed through the dialysis membrane to the buffer on the other side of the membrane and this was pumped through an enzyme reactor containing immobilized urease. The conductivity change related to the solution concentration was detected and recorded.

Comparative studies of the flow-through and flow-injection systems and two dialysers of different areas indicated that the best system is the flow-injection system with a large dialyser area (1.5 x 298 mm^2). A linear relationship between the changes in conductivity and urea concentrations of this system was obtained in the concentration range 0.5 - 10 mM (r^2 = 0.9955). The analysis time was approximately 25 min, and the life time of the enzyme reactor was more than 260 h operation time (used intermittently over 6 months). Good agreement was obtained when the urea concentrations of human serum samples were determined by the enzyme-sensor system compared to the conventional methods (Berthelot and Fearon reactions). These were statistically shown using the regression line and the Wilcoxon signed rank tests.

Acknowledgments

The completion of this thesis would be quite impossible without the help of many people, whom I would like to thank.

I express my sincere thanks to my advisors Associate Professor Dr Proespichaya Kanatharana and Associate Professor Dr Panote Thavarungkul for their advice and suggestions through out the course of this work.

I would also like to thank:

Ajarn Punnee Asawatreratanakul for her advice about the enzyme;

Songklanagarin Hospital for providing the serum samples and the results of the Berthelot reaction;

The lecturers of the Department of Chemistry, Prince of Songkla University for instructing and providing me with the knowledge which is useful for my thesis and to my future;

The examination committee members of this thesis for their valuable time;

Staffs of the Department of Chemistry and the Department Physics for their help in some technical aspects of this thesis;

and lastly

My friends in the Biophysics Research Unit; Biosensors and Biocurrents who help in many ways.

Contents

	Page
Thai abstract	(1)
English abstract	(2)
Acknowledgments	(3)
Contents	(4)
List of Tables	(6)
List of Figures	(8)
Chapter	
1 Introduction	1
1.1 Introduction	1
1.2 Literature review	3
1.3 Objectives of the research	13
1.4 Benefits	14
1.5 Outline of the research	14
2 Materials and Methods	15
2.1 Materials	15
2.2 Apparatus	16
2.3 Urea conductivity sensor	17
2.4 Immobilization of urea	18
2.5 Instrumentation	20
2.6 Data analysis	23
2.7 Optimization of the flow systems	24
2.8 Life time of urea standard solution	29
2.9 Buffer concentration	30
2.10 Stability of immobilized enzyme	30

Page

2.11 Determination of urea in serum samples 30

Chapter

3 Results and Discussion 35

3.1 Characteristics of the flow biosensor response 35

3.2 Optimization of the flow systems 36

3.3 Lifetime of urea standard solution 60

3.4 Buffer concentration 63

3.5 Stability of immobilized enzyme 65

3.6 Determination of urea in serum samples 68

4 Conclusion 80

References 83

Vitae 89

List of Tables

Table		Page
1.	Wilcoxon signed rank test	34
2.	Responses of a flow-through urea sensor system at different flow rates of the buffer line.	37
3.	Responses of a flow-through urea sensor system at different flow rates of the sample line.	40
4.	Responses of a flow-through urea sensor system at different sample volume.	42
5.	Responses of a flow-injection urea sensor system at different flow rates of the buffer line.	44
6.	Responses of a flow-injection urea sensor system at different flow rates of the sample line.	46
7.	Responses of a flow-injection urea sensor system at different sample volume.	48
8.	Comparison of the results obtained with the flow-through and the flow-injection systems at their optimum conditions.	51
9.	Responses of a flow-injection urea sensor system with the large dialyser at different flow rates of the buffer.	54
10.	Responses of a flow-injection urea sensor system with the large dialyser at different flow rates of the sample.	55
11.	Responses of a flow-injection urea sensor system with the large dialyser at different sample volume.	56
12.	The conductivity change of the flow-injection system with the small dialyser.	58
13.	The conductivity change of the flow-injection system with large dialyser.	59

Table Page

14. Response to urea standard solutions stored at room temperature (23°-30°C) during a 7-day period. 60
15. Response to urea standard solutions stored at 4°C during a 7- day period. 62
16. Responses of a flow-injection urea sensor system at different buffer concentrations. 64
17. Response of a flow-injection urea sensor system at different operation time of enzyme reactor. 66
18. Urea concentration obtained by Bethelot raction. 68
19. The response to urea calibration solutions of the coductometric urea biosensor system. 69
20. Urea concentration in serum sample obtain with conductometric urea biosensor system. 71
21. Absorption of urea standard solution obtained with Fearon reaction 72
22. Urea concentration in serum sample obtained with Fearon reaction 74
23. Application of the Wilcoxon signed rank test for the comparison of the concentration of ura in serum samples from the conductometric urea biosensor and the Berthelot reaction. 77
24. Application of the Wilcoxon signed rank test for the comparison of the concentration of urea in serum sample from the conductometric urea biosensor and Fearon reaction 79

List of Figures

Figure		Page
1.	Synthesis of urea from carbon dioxide and ammonium by urea cycle	1
2.	Urea and molecular structures relevant to the Fearon reaction	2
3.	The biosensor: the bio recognition of biological material produces a signal that is detected by a transducer.	17
4.	Immobilization methods for enzyme.	18
5.	Schematic diagram showing the flow-through system.	21
6.	Schematic diagram showing the dialyser.	21
7.	Block diagram of the detection unit.	22
8.	Schematic diagram showing the flow-injection system.	22
9.	A typical response of immobilized urease to urea measured as a direct current voltage signal that is related to the conductivity of the solution as recorded by the analytical system.	23
10.	Used of a regression line to compare two analytical methods.	33
11.	Response of immobilized urease to urea measured as the change in conductivity of the solution and recorded as a voltage signal by the analytical system.	35
12.	Response of a flow-through urea sensor system at different flow rates of the buffer line.	38
13.	Response of a flow-through urea sensor system at different flow rates of the sample line.	41
14.	Responses of a flow-through urea sensor system at different sample volume.	43
15.	Responses of a flow-injection urea sensor system at different flow rates of the buffer line.	45

Figure Page

16. Responses of a flow-injection urea sensor system at different flow rates of the sample line. 47

17. Responses of a flow-injection urea sensor system at different sample volume. 49

18. Comparison of the response obtained with the flow-through and flow-injection systems at their optimum conditions. 52

19. Responses of a flow-injection urea sensor system with the large dialyser at different flow rates of the buffer line. 54

20. Responses of a flow-injection urea sensor system with the large dialyser at different flow rates of the sample. 55

21. Responses of a flow-injection urea sensor system with the large dialyser at different sample volume. 56

22. Calibration curve of urea using the flow-injection system with the small dialyser 58

23. Calibration curve of urea using the flow-injection system with the large dialyser 59

24. Response to urea standard solutions stored at room temperature (23°-30°C) during a 7-day period. 61

25. Response to urea standard solutions stored at 4°C during a 7- day period. 62

26. Responses of a flow-injection urea sensor system at different buffer concentrations. 64

27. Response of a flow-injection urea sensor system at different operation time of enzyme reactor 67

28. Calibration curve of conductometric urea biosensor system 70

29. Calibration of urea standard solution obtained with Fearon reaction 73

Figure

Page

30. Correlation of the concentrations of urea in serum samples obtained from the conductometic urea the biosensor and the Bethelot reaction. 75

31. Comparison the concentration of urea in serum samples obtained from the conductometric urea biosensor and the Fearon reaction. 78

Chapter 1

Introduction

1.1 Introduction

Urea (H_2NCONH_2) is a white crystalline compound also known as carbamide and was isolated from urine in 1773 by Rcuelle. It was one of the first compounds to be artificially produced (Taylor and Vadgama, 1992). The body uses urea production in the liver to remove potentially toxic products of nitrogen metabolism as shown in Figure 1 (Rock, *et al.*, 1986).

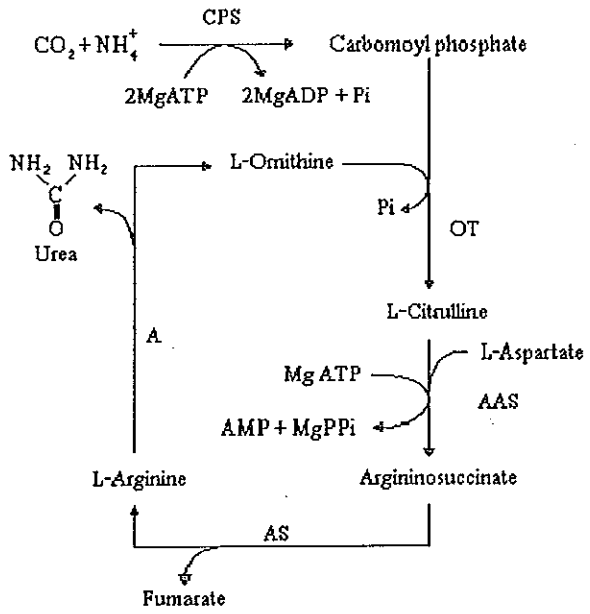

Figure 1. Synthesis of urea from carbon dioxide and ammonium by urea cycle.

CPS = carbamoyl phosphate synthetase;

OT = ornithine transcarbamoylase;

AAS = argininosuccinic acid syntase;

AS = argininosuccinase;

A = arginase

(Redrawn from Taylor and Vadgama, 1992; Lehninger, 1987)

The urea content of blood serum depends on protein catabolism and nutritive protein intake and is regulated by renal excretion (Scheller and Schubert, 1992). Excretion into urine is the major route for disposal of urea. Less than 10% is lost via the skin and gastrointestinal tract (Taylor and Vadgarma, 1992). Frequently, in a diseased state, in the case of renal failure, the patient may be unable to excrete urea with the consequence that high urea concentrations appear in the blood stream (Lienado and Rechnitz, 1974). Therefore, the serum concentration of urea provides information on kidney function. This is why the determination of urea is one of the most frequent analyses in routine clinical laboratory work.

Several methods have been described for the quantification of urea and these can be grouped into direct or indirect methods (Mascini and Guilbault, 1977). In the direct methods, urea is reacted by a color reagent to give a color solution, which is then measured spectrophotometrically (Lienado and Rechnitz, 1974). However, these have the disadvantages of non-ideal Beer's law behavior, nonspecific, the process requires some heat treatment, and some of the reagents are noxious (Lienado and Rechnitz, 1974).

Indirect methods generally involve monitoring of the products resulted from the catalytic reaction of urea by the enzyme urease (EC 3.5.1.5). For example, NH_3 which is produced by the hydrolysis of urea by free enzyme urease

$$(H_2N)_2CO + H_2O \xrightarrow{\text{UREASE}} 2\,NH_3 + CO_2$$

is estimated in Nesslerization and Bethelot reaction methods (Taylor and Vadgama, 1992). However, these two methods use urease as a reagent and this significantly increases the costs of analysis. Therefore, immobilized enzymes are now applied more frequently as they can be used several times. Immobilized enzyme has also been used in combination with a detector sensitive for the products of the biocatalytic process forming a urea biosensor, and several biosensors for urea have been reported

(Jurkiewicz *et al.*, 1996; Lee *et al.*, 2000; Liu *et al.*, 1995; Thavarungkul and Kanatharana, 1994).

Urease-based biosensors use various techniques to monitor urea. These include UV-visible spectrophotometry, potentiometry with the application of pH electrode, NH_3 electrode, CO_2 electrode, ammonium ion-selective electrode, ammonium ion-selective field effect transistor, coulometry, amperometry and methods using fiber-optics (Jurkiewicz *et al.*, 1996; Lee *et al.*, 2000; Liu *et al.*, 1995). However, there are several disadvantages associate with these methods. For examples, ion selective electrodes are vulnerability to the interference of other ions in the sample solution and also have high detection limit. Detection of NH_3 and CO_2 have relatively slow response time and time-consuming. UV-visible spectrophotometry needs extensive sample pretreatment and show poor precision (Scheller and Schubert, 1992; Watcerz *et al.*, 1998). Therefore, an alternative method is required.

In this work we propose the use of a conductivity meter as a transducer for a urease-based biosensor. Since the catalysis reactions of urea by urease produce charged products as shown

$$(H_2N)_2CO + 3\ H_2O \xrightarrow{\text{urease}} 2NH_4^+ + HCO_3^- + OH^- + \Delta H\ (-61\ KJ\ mol^{-1})$$

The conductivity of the solution should increase and the effect should be possible to detect using conductivity electrodes. The responses of enzyme to urea concentrations can then be quantified as the changes in conductivity and the relationship between urea concentrations and the changes in conductivity can be determined.

1.2 Literature review

Determination of urea has numerous applications. In pharmaceutical industry where urea is used as a component of many ointments, its level in these products must

be strictly controlled. In food industry the control of food quality (mainly farm products) also includes determinations of urea, for example in milk. The production of fertilizers and environmental protection are other areas for application of urea determination. For example, the presence of urea in river or ground waters gives the evidence of contamination with sewage. The high content of urea is one of the reasons for algae blooming. Moreover, the level of urea can be used to estimate the time when contamination has occurred (Walcerz *et al.*, 1998).

In human body, urea is the most important end product of protein degradation. The body uses urea production to remove ammonia which is a potentially toxic product of nitrogen metabolism (Taylor and Vadgama, 1992).

Ammonia is a product of amino acid metabolism and the major source of circulating ammonia is the gastrointestinal tract. It is neurotoxic, possibly due to its effect on the glutamate dehydrogenase pathway causing a reduction in the α-ketoglutarate available to the citric acid cycle. Animal must, therefore, remove ammonia from the body. This may be direct (ammonotelic) as in fish, via uric acid (uricotelic) as in frogs, or via urea (ureotelic) as in mammals.

Urea synthesis (Figure 1) from ammonia occurs only in the liver, by means of a cyclical process in which ornithine acts as the initial ammonia carrier, and is regenerated following production of the urea molecule. The second amino group of urea is derived from aspartate. Three moles of ATP are consumed for each mole of urea generated, yielding 2 moles of ADP and 1 mole of AMP (Taylor and Vadgama, 1992).

Excretion into urine is the major route for disposal of urea. Less than 10% is lost via the skin and gastrointestrinal tract. Urea is freely filtered at the glomerulus and tubular cells do not actively reabsorb or secrete urea. However, it is highly diffusible and approximately 50% leave the tubular lumen and returns to plasma, via the renal interstitial. When urine flow is rapid this diffusion is less.

Historically urea was used to evaluate renal function. A widely accepted reference interval for serum urea is 2.3-8.3 mM, derived from young men on a normal diet (Lum and Leal-Khouri, 1985). However, other factors such as age, sex, pregnancy and diet are known to influence serum urea (Taylor and Vadgama, 1992). Despite its limitations urea levels are still used as a rough predictive index of symptotic renal failure and as a diagnostic aid in distinguishing among the various causes of renal insufficiency (Allston, 1993).

Methods for determining urea are classified into two groups: direct methods and indirect methods (Lienado and Rechnitz, 1974).

Direct Methods

In most direct methods, urea is reacted by the color reagents to give a color compounds, which is then measured spectrophotometrically (Lienado and Rechnitz, 1974). Fearon was the first to show that urea and other compounds having the R_1NH-$CONHR_2$ (when R_1 is H or a single aliphatic radical and R_2 is not an acyl radical) react with diacetyl monoxime in the presence of strong acid and an oxidizing agent to produce a chromogen (Taylor and Vadgama,1992). The reaction mechanism is shown in Figure 2

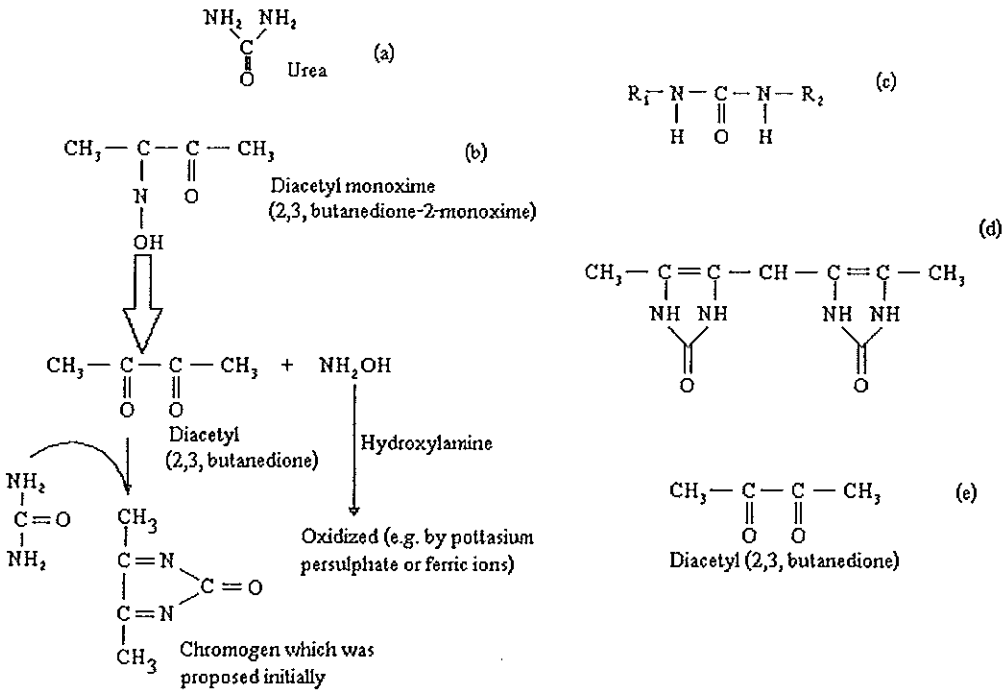

Figure 2. Urea and molecular structures relevant to the Fearon reaction.

(a) Urea. (b) Reaction scheme. (c) General form of reacting species;

R_1 = H or single aliphatic radical; R_2 = not an acyl radical.

(d) Putative chromogen. (e) Diacetyl.

(Redrawn from Taylor and Vadgama, 1992)

Diacetyl (2,3 butanedione (Figure 2 (e)) produces an identical color on reaction with urea and it is assumed that diacetyl monoxime hydrolyses to diacetyl before combining with urea. Ormsby applied the Fearon reaction to measure blood and urine urea in a protein-free solution in 1942 (Taylor and Vadgamar, 1992). Heat and strong acidic conditions were used to generate the chromogen which has a strong absorption peak at 540 nm, and it was noted that while many substituted ureas gave a red colour, only urea produced a yellow one. Oxidizing agents such as phosphoric acid with sulfuric acid were found to increase the colour intensity, and it is believed that this effect is due to their destruction of the hydroxylamine produces when diacetyl

monoxime hydrolyses to diacetyl. Potassium persulphate has been widely used as an oxidizing agent for this purpose. In order to avoid the use of oxidizing agent, diacetyl rather than diacetyl monoxime was proposed as the reagent, but diacetyl is less stable and methods using it were regarded as problematic and have been fallen into disuse.

The disadvantages of the Fearon methods include: color develops rapidly and fades rapidly; the color is photosensitive; the color does not follow Beer's law with either a filter photometer or spectrophotometer; the unpleasant odor and irritant fumes of the reagents make it advisable to work in a fume hood; with diacetyl monoxime the time of heating for maximal color development is dependent on the urea concentration; and the reaction is not completely specific (Lienado and Rechnitz, 1974; Marsh *et al.*, 1965; Taylor and Vadgama, 1992).

Several instrument manufacturers have employed another method using the reaction of o-phthalaldehyde with primary amines to quantify urea. The isoindoline product of the reaction is coupled to a complex quinoline to form a chromogen that is monitored at 510 nm (Taylor and Vadgama, 1992). The Ames Seralyser (Ames Division, Miles Laboratories, Inc., Elkhart, IN) combines the o-phthalaldehyde reaction with their dry paper strip technology. A cation-exchange matrix is added to the reagent layer to catalyze the reaction between the isoindoline and the quinoline to form the chromogen. The reaction is monitored by reflectance spectrophotometry. Unlike the Fearon method, these reactions do not require incubation at high temperatures. The method remained unpopular because of the caustic nature of some of the reagents, despite the stability, cheapness and speed of the reaction. Sulphonamide drugs are a widely recognized source of positive interference.

There are some other direct methods, though unsuitable for routine clinical use, might be used as research tools, especially for small sample volume (Lienado and Rechnitz, 1974). For examples, high performance liquid chromatography (HPLC) method has also been used for the determination of urea, using an amino stationary

Nesslerization methods have been replaced by the indophenol reaction of Bertholet that is about 10 times more sensitive for ammonia than Nesslerization (Koncki *et al.*, 1999). Bethelot described in 1859 the reaction of phenol with ammonia in the presence of hypochlorite to produce a blue colour (Taylor and Vadgamar, 1992).

Modifications to the Berthelot method have been described by several authors (Gordon *et al.*, 1978; Tabacco *et al.*, 1979), including the replacement of phenol with salicylate and replacement of hypochlorite with dichloroisocyanurate (Searcy, 1969). These changes result in the formation of a stable green coloured product. Gordon *et al.* (1978) reviewed conditions for the Berthelot reaction, comparing the suitability of phenol and salicylate. Although less expensive than salicylate, the phenol reagent was considered less stable, more deliquescent and more sensitive to deviations in pH.

Adaptations of the Berthelot reaction for continuous flow and discrete automatic analysers have been developed. Urease has been immobilized on the inner surface of tubes through which samples flow and these tubes have been used with the Bethelot method for determination of urea. Attachment of enzyme using alkylated nylon has been described (Chirillo *et al.*, 1979; Sundaram *et al.*, 1978) for use in continuous flow methods and stability is claimed over several thousand analyses. Another approach, urease has been immobilized on glass beads via poly γ-methyl L-glutamate with no change in optimal pH or loss of activity (Talor and Vadgama, 1992). A major advantage is reduction in cost through reusability of the expensive enzyme and reduced reagent preparation time, with analytical performance comparable to dissolved urease methods. The use of immobilized enzyme together with an appropriate measuring device has become part of a technique called biosensor.

Biosensor is an analytical technique that has been developed since 1962 by Clark and Lyons. It is the device which incorporates a biological sensing element with the signal transducer, to give a sensing system specific for the target analyte (Cooper and Mcneil, 1990).

Since the first urea biosensor was prepared by Guilbault *et al.*, (Lienado and Rechnitz, 1974) the development of urea biosensors using immobilized urease has attracted continue interest, and various type of urea biosensors have been reported (Adams and Carr, 1978; Jurkiewicz *et al.*, 1996; Koncki *et al.*, 1999). In the urea biosensor, the urease catalytically converts urea into several products (reactions (1) - (4))

To monitor the enzymatic reaction, various techniques, such as UV-visible spectrophotometry (Liu *et al.*, 1995), potentiometry with the application of pH electrode (Knocki *et al.*, 1999), ammonium ion-selective electrode (Eggenstein *et al.*, 1999) and ammonium ion-selective field effect transistor (Lee *et al.*, 2000), coulometry (Ivnitskil and Rishpon, 1993), amperometry (Adams and Carr, 1978), and conductometry (Thavarungkul and Kanatharana, 1994) have been employed.

Most urea sensors are based on potentiometry. Guilbault and Nagy (1973) used NH_4^+ sensitive electrode based on nonactin to determine urea in urine sample. A sensor for urea measurement in blood was later developed in 1984 by Tokinaga *et al.* (Scheller and Schubert, 1992) where two NH_4^+ sensitive electrodes contained nonactin in PVC membranes were integrated in a flow injection analysis device in a differential circuit. However, these electrodes have poor selectivity, and potassium and sodium may interfere with the signals (Watcerz *et al.*, 1998). This is a serious disadvantage imposing severe limitation on the use of this electrode for measurements in biological fluids, for example, serum. Therefore, the elimination of these interference is important in the determination of urea in biological fluids when enzymatic sensor based on ammonium sensitive electrode are applied.

The pH increase caused by urea hydrolysis can also be indicated by using pH sensitive glass or metal oxide electrode (Scheller and Schubert, 1992). The major problem of pH-sensing electrodes is that the sensor response is strongly dependent on the buffer capacity of the sample because the pH change produced in the course of the

enzyme-catalysed reaction can be suppressed by buffer used, which lead to a narrow dynamic range and a loss in sensor sensitivity (Eggenstein *et al.*, 1999).

Potentiometric gas sensors for the reaction products, NH_3 and CO_2, have also been employed (Hanson and Ruzicka, 1974). Since these measurement are based on gas diffusion through a hydrophobic membrane, no direct disturbances by sample constituents occur. A major drawback of these sensors is their long response time, which is due to the slow diffusion of the gases. Since it takes several additional minutes to reach a new baseline after each measurement, only a few samples can be processed per hour (Watcerz *et al.*, 1998). A further disadvantage of potentiometric gas sensors is the difference between the pH optima of electrode and urease. Thus, NH_3 electrodes are operated at pH around 8 while the optimal pH of the urease reaction is at pH 7 (Scheller and Schubert, 1992).

The advantage of amperometric electrode, such as the greater sensitivity and precision and the lower measuring time, have prompted several research groups to study the adoption of this measuring principle to the assay of urea (Adams and Carr, 1978). Altogether many different approaches have been investigated. The linear dependence of the oxidation current of hydrazine on OH^- concentration has been the most thoroughly studied in the urease-catalysed hydrolysis of urea (Scheller and Schubert, 1992). Between pH 5 and 9 the anodic oxidation current of hydrazine at +100 mV vs SCE depends linearly on OH^-

$$N_4H_4 + 4OH^- \longrightarrow N_2 + 4H_2O + 4e^-$$

The sensitivity depends on the initial pH. The response time of the amperometric urea sensor was 7-15 s, the sample frequency being 40/h and the linear measuring range was 0.8-50 mM. The excellent precision was demonstrated by a CV (coefficient of variation) below 1%. Since this measuring principle, like potentiometric pH electrodes,

is subject to the influence by different pH values and buffer capacities of the sample, therefore, the differential measurement between the enzyme electrode and the enzyme-free electrode was needed to give the true urea concentration in serum.

Thermal biosensors, for example an enzyme thermistor, are interesting alternatives to these devices because they employ a universal detection principle, namely heat (Xie *et al.*, 1995). The amount of reacted substrate is related to the heat produced through the specific enthalpy, ΔH_r, of the reaction (Bjarnason *et al.*, 1998). The first application of thermal sensor on the urea determination has been described by Mosbach in 1976 (Rich *et al.*, 1979). As practically all-biological reactions are exothermic, this principle is applicable as long as suitable specificity is obtained in the enzymatic reaction. A drawback of this device is the non-specific heat effects from mixing, change in pH, viscosity and ionic strength can also produce signals (Bjarnason *et al.*, 1998).

Conductometric has become an alternative and promising way to detect the reaction of urease (Lee *et al.*, 2000; Mikkelsen and Rechnitz, 1989; Sheppard and Rechnitz, 1995; Thavarungkul and Kanatharana, 1994). Urease immobilized to solid support catalyzed the hydrolysis of urea, in an overall reaction leading to the formation of ammonium, bicarbonate and hydroxide ions:

$$NH_2CONH_2 + 3H_2O \xrightarrow{\text{UREASE}} 2NH_4^+ + HCO_3^- + OH^-$$

Here, the rate of increase of conductivity is related to the urea concentration in the sample.

Five properties that, separately or in combination, allow the application of conductometric method to enzymatic reaction are (1) the generation of ionic groups; (2) the separation of unlike charge; (3) proton migration; (4) changes in degree of association of ionic groups resulting from chelation; and (5) changes in the sizes of

charge-carrying group (Mikkelsen and Rechnitz, 1989). Because all charge-carrying species are detected simultaneously, conductometric methods are relatively nonselective. Buffer of low ionic strength must be used for the detection of low levels of substrate, since detection limits are ultimately controlled by the ratio $\Delta G/G$, where G is the conductance of the medium and ΔG is the conductance change that results from the enzymatic process. This same limitation is expected to apply to conductometric biosensors. Ideal chemical systems with which to test conductometric transducers are those processes possessing the highest percentage change in total conductance (conductance coefficients) under identical conditions. Factors (1) and (2) above give rise to the largest conductance coefficients and should be presence in the systems of choice.

The interest in the conductometric biosensor stems to a large extent, from the relative simplicity (no reference electrode needed) and easy fabrication of the biosensor. In this work, a conductometric biosensor using immobilized urease in a flow system which satisfied factors (1) and (2) mentioned above, has been developed. The system's performance has been tested in the determination of urea levels in human serum compared with Fearon reaction and Berthelot methods.

1.3 Objectives of the research

1. To develop and evaluate the performance of a flow-through urea biosensor system.
2. To develop and evaluate a flow-injection analysis urea biosensor system and compared this with the flow-through system.
3. To use the biosensor system to analyze urea concentration in human serum and compared with some standard methods.

1.4 Benefits

It is expected that the proposed conductometric urea biosensor will be used as an alternative method to determine urea, which is low cost, simple to use and correlate well with standard methods.

1.5 Outline of the research

1. Immobilized urease on alkylamine glass beads
2. Optimize the operating conditions of the-flow through biosensor system, such as flow rate, sample volume, *etc.*
3. Optimize the operating conditions of the biosensor in a flow-injection analysis system and compared this with the flow-through system.
4. Test the biosensor system by determining urea in human serum samples and compare the results with those obtained using Fearon reaction and Berthelot Methods.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Biosensor technique

- Urea ($NH_2(CO)NH_2$, Analytical Grade: Mallinckredt, USA.)
- Urease (amidohydrolase EC 3.5.1.5 Type IV: from jack beans, 74,000 units/g solid: Sigma, USA.)
- Glutaraldehyde 25% ($C_5H_8O_2$, Biological Grade: Electron Microsopy Science, USA.)
- Ethanolamine (C_4H_7NO, AR Grade: Merck, Germany.)
- Alkylamine glass beads (from porous glass beads, mean diameter 41μm, mean pore diameter 20 nm, Eka Noble AB, Sweden.)
- Sodium cyanoborohydride (CH_3BNNa, AR Grade: Fluka, Switzerland.)
- Sodium azide (NaN_3, AR Grade: Merck, Germany.)
- Glycine (H_2NCH_2COOH, AR Grade: Merck, Germany.)
- Sodium dihydrogenphosphate dihydrate ($NaH_2PO_4.2H_2O$, AR Grade: Merck, Germany.)
- Disodium hydrogenphosphate dihydrate ($Na_2HPO_4.2H_2O$, AR Grade: Ferar, Germany.)
- Sodium hydroxide (NaOH, AR Grade: Merck Germany.)
- Sodium Chloride (NaCl , AR Grade: BDH, England.)
- Hydrochloric acid 36.5-38%(HCl, AR Grade: BDH, England.)

2.1.2 Fearon reaction technique (BUN Sigma Kit)

- BUN acid reagent (ferric chloride in phosphoric and sulfuric acids)(Reagent Grade: Sigma, USA.)
- BUN color reagent (diacetyl monoxime, 0.18% (w/v), and thiosemicarbazide) (Reagent Grade: Sigma, USA.)
- Urea at a urea nitrogen level of 30 mg/dL (10.7 mmol/L) with benzoic acid as preservative (Reagent Grade: Sigma, USA.)
- Urea at a urea nitrogen level of 150 mg/dL (53.5 mmol/L) with benzoic acid as preservative (Reagent Grade: Sigma, USA.)
- Trichloroacetic acid (TCA) 3% (Reagent Grade: Sigma, USA.)

2.2 Apparatus

2.2.1 Biosensor technique

- Peristaltic pump (Minipuls 2, Gilson, France.)
- Dialyser
- Cellulose ester membrane (Spectra/por 1, MWCO 6,000)
- Sample injector (Model 7125 Syringe Loading sample injector, Rheodyne, USA.)
- Exmire microsyringe (Code No. MS*R500, Ito Corporation Fuji, Japan)
- Conductivity meter
- Chart recorder (Single channel Model 155, Linear Instrument Company, USA.)
- Sample rocker

2.2.2 Fearon reaction technique

- Spectrum 351 Spectrophotometer (Tran Orchid Consulting, Inc.,USA.)
- General Laboratory Centrifuge (GLC-2, USA.)

2.3 Urea conductivity sensor

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 (Cooper and Mcneil, 1990). The utilization of the biological element capitalizes on the unique specificity of biological molecules for target species (Figure 3). The transduced signal in a biosensor is due to the reaction between the biorecognition molecule and the target analyte. The use of this indirect means of assay means that chemically similar solution species can be identified by their biospecific reaction with an immobilized biomolecule such as an enzyme, antibody, *etc.*

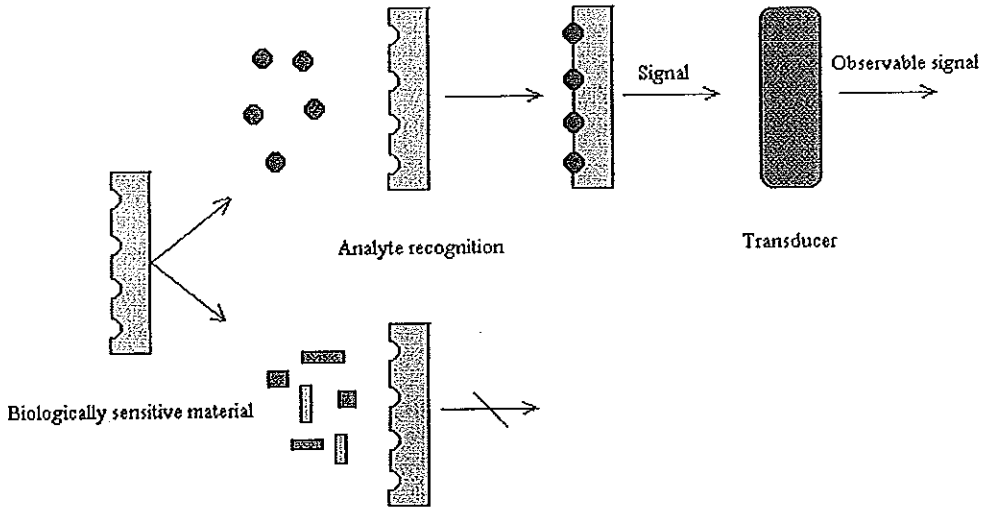

Figure 3 The biosensor: the bio-recognition of biological material produces a signal that is detected by a transducer.

In this work the enzyme urease was used as the biological material specific to urea. Urease catalyses the hydrolysis of urea to ammonia and carbondioxide. Ammonia and carbondioxide are further reacted to yield ammonium and hydrogen carbonate ions as shown in the following reaction schemes:

$$(H_2N)_2CO + H_2O \xrightarrow{\text{UREASE}} 2\,NH_3 + CO_2 \quad \text{---------(1)}$$

$$2\,NH_3 + 2\,H_2O \longrightarrow 2\,NH_4^+ + 2\,OH^- \quad \text{---------(2)}$$

$$CO_2 + H_2O \longrightarrow HCO_3^- + H^+ \quad \text{---------(3)}$$

The overall reaction is

$$(H_2N)_2CO + 3H_2O \xrightarrow{\text{UREASE}} 2NH_4^+ + HCO_3^- + OH^- \quad \text{---------(4)}$$

This enzyme-substrate reaction causes a change in the ionic strength of the solution under investigation, and this can be monitored by conductance measurements.

2.4 Immobilization of urease

Immobilized enzymes are defined as "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously" (Chinbata, 1978). Immobilization of enzyme is classified into carrier-binding, cross-linking and entrapping types, as shown in Figure 4.

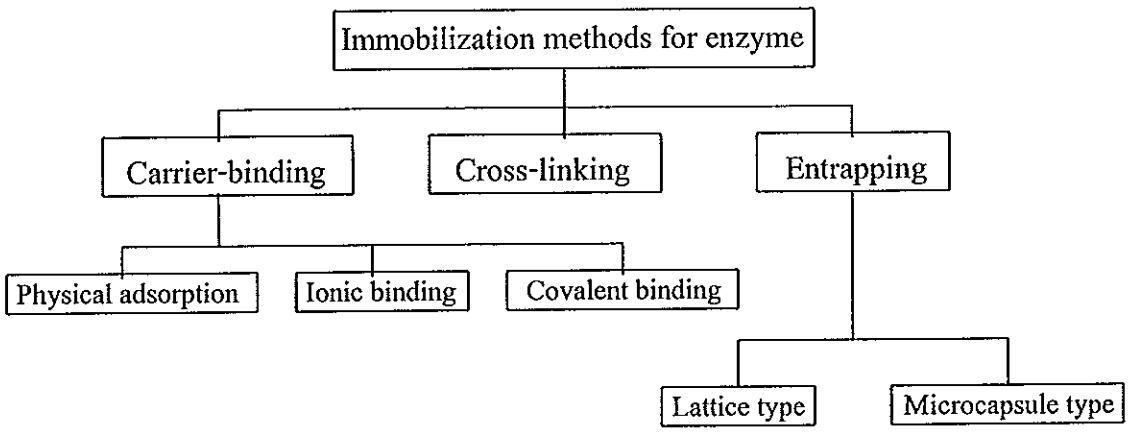

Figure 4 Immobilization methods for enzyme.

Carrier-binding is subdivided into physical adsorption, ionic binding and covalent binding, while entrapping is divided into lattice type and microcapsule type. In this work, covalent binding between urease and alkylamine glass beads was chosen.

The activation of alkylamine glass by glutaraldehyde to yield aldehyde glass was done by adding 0.4 g of alkylamine glass into 25 ml of 2.5% (v/v) glutaraldehyde in 0.05 M sodium phosphate buffer pH 7.0. 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 crosslink will occur. The crosslinked enzyme will decrease the overall activity by blocking pores and preventing passage of larger molecules.

To immobilize the enzyme, 100 mg of urease (7,400 unit) was dissolved in 5 ml of 0.05 M sodium phosphate buffer pH 7.0 and added to 1 ml (sedimented volume) of activated glass. The mixture was tumbled at room temperature (around 23°C). After 4-5 h, 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 h and was then washed with 500 ml of buffer. After this 25 ml of 0.1 M ethanolamine pH 8.0 was added and the reaction was allowed for another 2 h. 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 (inner diameter 4 mm, length 30 mm) to be used in the analytical process. When not used, the column was stored in 0.05 M sodium phosphate buffer pH 7.0 + 0.02% sodium azide at 4°C.

2.5 Instumentation

Two flow systems, flow-through and flow-injection systems, were studied in this work. The flow-through system (Figure 5) 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 system. For the flow-through system, sample solutions were introduced as pulses in the continuous flow of buffer (blank) by switching the tube between buffer and sample containers.

c) A separation unit, a dialyser (Figure 6), 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 membrane. Two dialysers with different diffusion areas, 1.5 x 49 mm^2 and 1.5 x 298 mm^2, were tested.

d) A reaction unit, a unit that the reaction will occur. In this work we used an enzyme reactor where the immobilized urease catalysed the hydrolysis of urea into charged products.

e) A detection unit (Figure 7), this consisted of a conductivity cell and a conductivity meter that monitored the change in the conductance of the solution due to the change of the charges in the solution. The signal was then recorded on a chart recorder.

The conductivity cell consists of two 10 mm long stainless steel tubes (outer diameter approximately 0.9 mm) glued to the ends of a glass tube (17 mm long, inner diameter 1.0 mm). The ends of the electrodes inside the glass tube are approximately 4 mm apart. A sine wave with a frequency of 1.6 kHz and an amplitude of 0.4 V was used. The alternating current response was converted to a direct current signal and was recorded. This voltage signal was linearly related to the solution conductance.

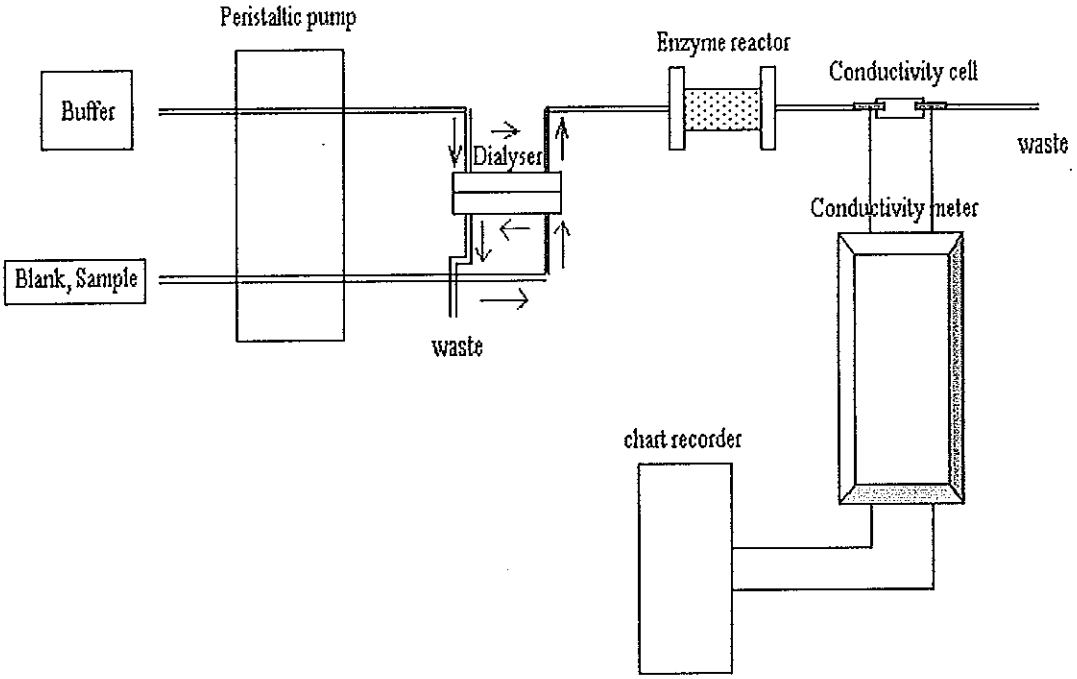

Figure 5. Schematic diagram showing the flow-through system.

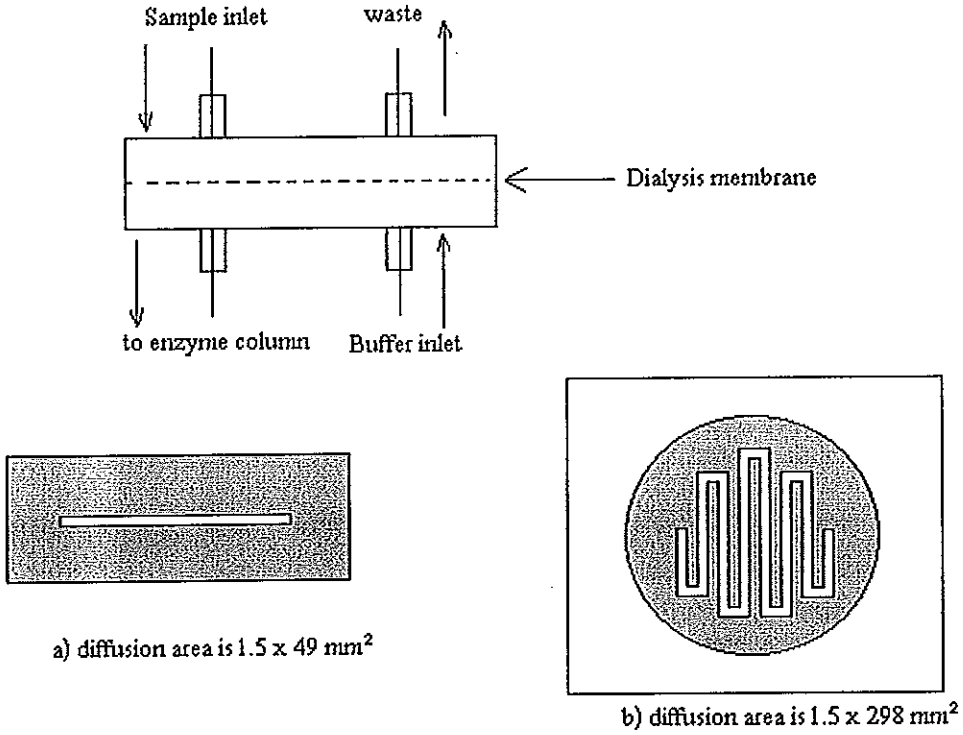

Figure 6. Schematic diagram showing the dialyser. Two dialysers with different diffusion area were tested, a) 1.5 x 49 mm^2 and b) 1.5 x 298 mm^2.

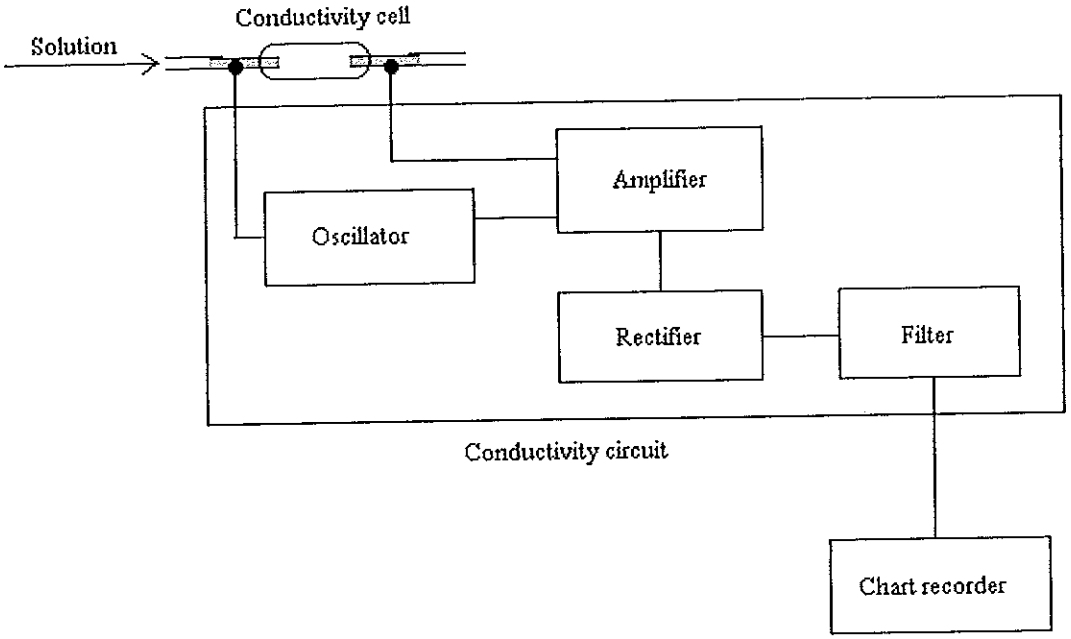

Figure 7. Block diagram of the detection unit.

(Redrawn from Thavarungkul and Kanatharana, 1994)

In the flow-injection system (Figure 8) the only different is the sample loading unit. In this case a specific volume of the sample was injected into an injection valve before introduced into the sample carrier buffer.

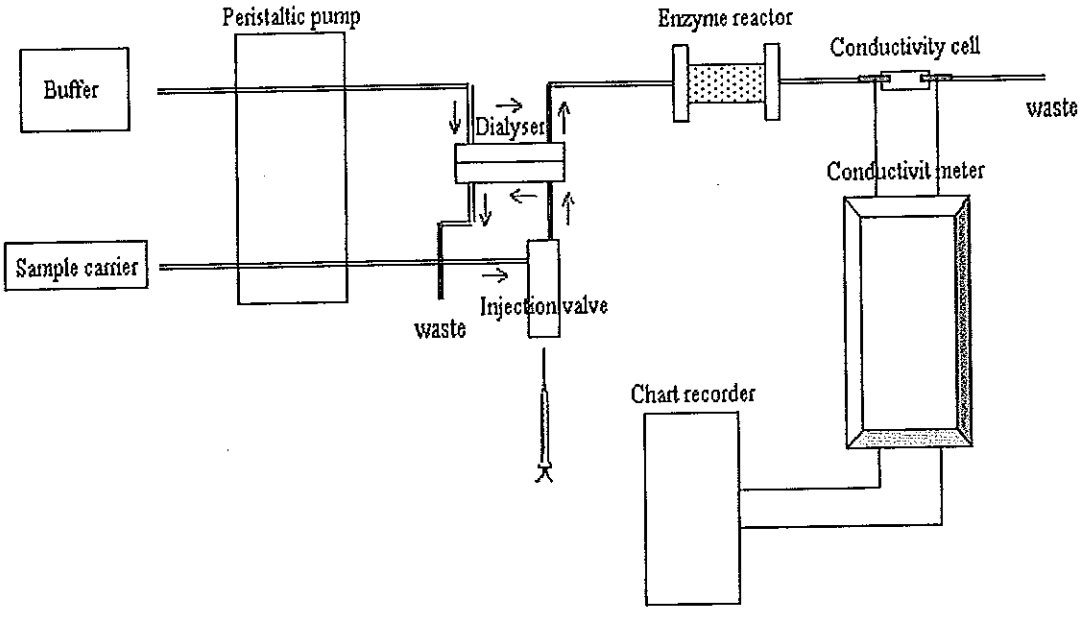

Figure 8. Schematic diagram showing the flow-injection system.

2.6 Data analysis

A typical recorder output has the form of a peak (Figure 9), 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 the sample and a 95% decrease of the flow response, is the analysis time (Jurkiewicz *et al.*, 1998). The height, width or area of the transient signal observed by the detector due to the passage of the sample contains the analytical information. So, there are several ways to interpret the readout. In this work we choose peak height to interpret raw data since it is easily identified and directly related to the detector response. For each concentration the average of three pulses was obtained.

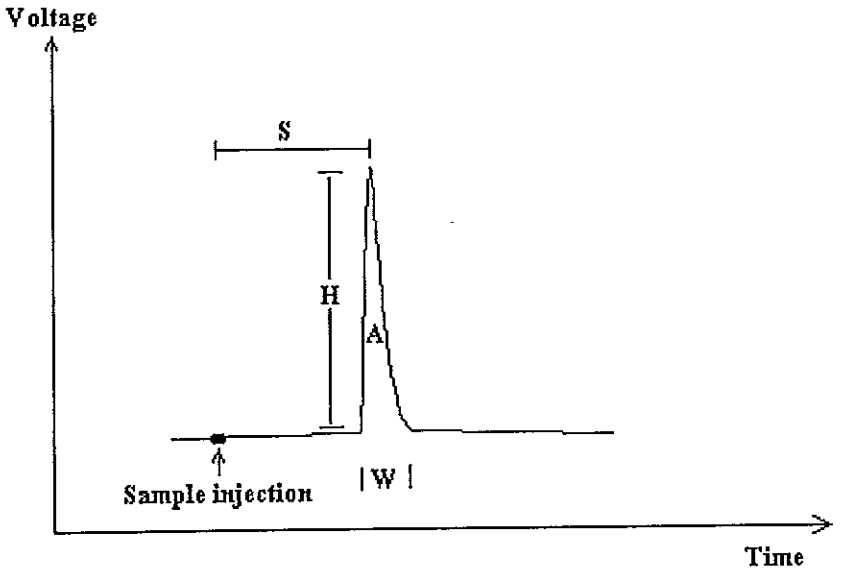

Figure 9: A typical response of immobilized urease to urea measured as a direct current voltage signal that is related to the conductivity of the solution as recorded by the analytical system.

2.7 Optimization of the flow systems

Initially the system were tested using the dialyser with a diffusion area of 1.5 x 49 mm^2 (Figure 6 (a)). All parameters of both the flow-through and flow-injection systems were optimized to obtain a maximum signal in the shortest period of time. To optimize the factors which may effect the result the uniparameter variation was used. This was done by changing a single variable while the others are kept constant. More advance optimization method were not applied as the system was not complex and the method used was simple, fast and adequate (Jurkiewicz *et. al.*, 1998).

The optimized variables of a flow system are the flow rate, the sample volume and the reactor length. In an earlier work of the Biophysics Research Unit: Biosensors and Biocurrent, Department of Physics, Faculty of science, Prince of Songkla University, the reactor characteristics had been optimized (Chaibundit, 1996) where a glass column reactor (4 mm inner diameter and 30 mm length) was chosen. So only the flow rate and the sample volume are optimized in this work.

2.7.1 The flow-through system.

In these studies the concentrations of urea being tested were 10, 20, 30, 40, 50, 60, 70, 80 and 90 mM.

2.7.1.1 Flow rate

In a flow system, the flow rate of the solution passing through the reactor and the detector is the main effective factor of the dispersion of the analyte particles, yield of the reaction and response of the detector. The dispersions that occur will affect the result of flow systems. 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 conductivity cell respectively. So the optimization of flow rate is necessary.

In this proposed system a dialyser 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.

The buffer used throughout the experiment, except when the buffer concentration was tested, was 0.05 M glycine-NaOH pH8.8. This was chosen because of its low conductivity. For the sample line the background solution was the same buffer plus 0.9% (w/v) NaCl. The salt was added so that the solution used will be isotonic to serum which would be later tested. All samples were prepared using this solution.

a) Flow rate of the buffer line

The effect of the flow rate of the buffer line was studied at 0.25, 0.40, 0.50, 0.60, 0.75 and 1.00 ml min^{-1}. The flow rate of the sample line was fixed at 0.50 ml min^{-1}. The sample pulse was 1 min (0.50 ml sample volume).

b) Flow rate of the sample line

The flow rates of the sample line were investigated at 0.10, 0.25, 0.50, 0.75 and 1.00 ml min^{-1}. The flow rate of the buffer line was 0.50 ml min^{-1} (optimum flow rate in (a))

2.7.1.2 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.

This was done by using 0.5 min (0.125 ml), 1.0 min (0.250 ml), 1.5 min (0.375 ml) and 2.0 min (0.500 ml) sample pulses. The flow rate of the buffer and sample lines were 0.50 and 0.25 ml min^{-1} respectively.

2.7.2 The flow-injection system

The difference between the two proposed systems is the sample-loading unit and this may change the characteristic properties of the flow system. So, the optimization of the flow parameters were necessary. The urea calibration solutions were 10, 30, 50, 70 and 90 mM.

2.7.2.1 The flow rates

a) Flow rate of the buffer line

Flow rates of the buffer line were 0.20, 0.25, 0.50 and 0.75 ml min^{-1}. On the other side of the membrane the sample line flow rate was 0.25 ml min^{-1} and the sample volume was 0.25 ml.

b) Flow rate of the sample line

This was investigated at 0.20, 0.25, 0.50 and 0.75 ml min^{-1}. The flow rate of the buffer line was set at 0.25 ml min^{-1} and the sample volume was 0.25 ml.

2.7.2.2 Sample volume

The effect of the sample volume was determined. The volumes tested were 0.15, 0.20, 0.25, 0.40 and 0.50 ml. The flow rates of the buffer and the sample lines were both 0.25 ml min^{-1}.

2.7.3 Comparison of the flow-through and flow-injection system

Two systems using the same urea standard solutions (1.0, 10, 20, 30, 40, 50, 60, 70, 80 and 90 mM) but different flow techniques (flow-through and flow-injection) were compared while working under optimum conditions. These are

	Sample Volume (ml)	Buffer line flow rate (ml min^{-1})	Sample line flow rate (ml min^{-1})
Flow-through	0.25	0.25	0.50
Flow-injection	0.25	0.25	0.25

The parameters considered 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 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.*

2.7.4 Optimization of the flow-injection system using a large area dialyser

The main aim of this work is to develop a biosensor system that can analyse the amount of urea in blood serum. In 2.7.3, it was found that the detection limit of the system was too high for the normal range of urea in human (2.3-8.3 mM). Therefore, a new dialyser with a larger area was tried since the diffusion area of a dialyser will have a direct effect on the amount of urea passing through the membrane into the reaction part. An increase of the dialyser diffusion area is expected to improve the analytical performance. In this work, the area was increased by 6 times from 1.5 x 49 mm^2 to 1.5 x 298 mm^2. This change may affect the optimum of the flow parameters and these were reoptimized. In these studies the concentrations of urea being tested were 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 , 9.0 and 10 mM.

2.7.4.1 Flow rates

a) Buffer line

The flow rates of the buffer line were investigated at 0.20, 0.25, 0.50 and 0.75 ml min^{-1}. The flow rate of the sample line was 0.25 ml min^{-1}. The sample volume was 0.25 ml.

b) Sample line

The flow rates of the sample were studied at 0.20, 0.25, 0.50 and 0.75 ml min^{-1}. The flow rate of the buffer line was 0.25 ml min^{-1}. The sample volume was 0.25 ml.

2.7.4.2 Sample volume

The effect of the sample volume was determined. The volumes tested were 0.15, 0.20, 0.25 and 0.40 ml. The flow rates of the buffer and sample lines were both 0.25 ml min^{-1}.

2.7.5 Comparison of the small and large dialysers

Systems using different dialysers were compared by considering sensitivity, linear range, precision, limit of detection and analysis time.

The optimum parameters were found to be the same for both dialysers i.e. optimum flow rates of the buffer and sample lines were 0.25 ml min^{-1} and optimum sample volume was 0.25 ml, and these were used to test the systems. The urea calibration solutions used were 0.5, 1.0, 5.0, 10, 20, 30, 40, 50, 60, 70, 80 and 90 mM for the small dialyser. For the large dialyser, the concentrations of urea being tested were lower since more urea molecules could pass through the dialysis membrane. The concentrations tested were 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10 mM.

2.8 Life time of urea standard solution

Urea hydrolysis can slowly occur by itself and in condition of bacterial contamination even with refrigerated storage (Taylor and Vadgama, 1992). This may effect the result of an experiment which take a long period of time. Therefore, the stability of the standard urea solutions needs to be investigated. This was done by monitoring the calibration curve of standard urea solutions (5, 10, 20, 30, 50 and 60 mM) which were stored at room temperature and at 4°C every day for 7 days. The flow-through system under the optimum condition (2.7.3) was used for this experiment.

2.9 Buffer concentration

Conductometric monitoring of enzyme-substrate reactions requires that the conductivity change can be discriminated from the conductivity of the background buffer. Therefore, a low concentration buffer is preferred. However, if the concentration is too low, buffer capacity of the solution is too small, and may result in the change of the local pH during the reaction (Mikkelsen and Rechnitz, 1989). Thus, it

is necessary to find the optimum buffer concentration that will suit the system. This was studied by using 0.005, 0.010, 0.050 and 0.100 M of glycine-NaOH buffer pH 8.8.

Concentrations of standard urea solutions were 0.5, 1.0, 5.0, 10, 20, 30, 40 and 50 mM. The flow-injection system with small dialyser was used in this study and the other tested conditions were as in 2.7.3.

2.10 Stability of immobilized enzyme

The long-term stability of the immobilized enzyme was tested intermittently over a period of 6 months (263 h operating time) by monitoring its response to urea standard solutions. Sensitivity of the calibration curve were used as indicating factors of the enzyme reactor.

2.11 Determination of urea in serum samples

To demonstrate the use of the conductometric urea biosensor the system was tested using the serum samples obtained from Songklanagarind Hospital, Prince of Songkla University. The same samples were analysed by UV-visible spectrophotometry using the commercially available blood urea nitrogen (BUN) test kit and the Bethelot reaction (the results obtained by Songklanagarind Hospital).

2.11.1 Berthelot reaction

The serum samples tested by Songklanagarind Hospital were by autoanalyzer (Hitachi, model 717) Berthelot reaction. Urea is hydrolyzed in the presence of water and urease to produce ammonia and carbondioxide. In this modified Bethelot reaction the ammonium ions react with hypochlorite and salicylate to give a green dye. The increase in absorbance at 578 nm is proportional to the urea concentration in the sample.

2.11.2 Conductometric urea biosensor

Before the urea was measured, the serum samples were diluted using 0.05 M glycine-NaOH buffer pH 8.8 (contained 0.9% (w/v) NaCl) at a serum : buffer ratio of 1 : 9.

To calibrate the system, standard urea solutions (0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 mM prepared in 0.05 M glycine-NaOH buffer pH 8.8 + 0.9% (w/v) NaCl) were injected into the system. The flow-injection system with a large dialyser was chosen because of its low limit of detection. The flow rates of the buffer and sample lines were both 0.25 ml min^{-1} and the sample volume was 0.25 ml. The calibration curve was prepared by plotting the conductivity change Vs corresponding urea concentration (mM). The sample solutions were then injected into the system. The change in the conductivity of each sample was used to calculate the urea concentration from the calibration done prior to the test.

2.11.3 Blood urea nitrogen (BUN) test kit (Sigma, procedure No 535)

This method is based on Fearon reaction which uses the direct interaction of urea with diacetyl monoxime.

Standard solutions of urea (blank, 5.0, 10, 15 and 20 mM) were prepared by diluting the urea nitrogen standard solution (catalog No 535-150) with distilled water. For each concentration 0.02 ml of the standard solution, was added to 3.00 ml of BUN acid reagent and 2.00 ml of BUN color reagent and mixed thoroughly. The mixture was placed in boiling water for exactly 10 minutes. It was then removed and placed in cold tap water for 3-5 minutes. The absorbance of the solution was recorded at 520 nm. The plot of the absorbance Vs urea concentration (mM) was used as the calibration curve.

To estimate urea concentration in serum samples, the proteins in the samples were precipitated by adding 1.80 ml of cold 3% trichloroacetic acid to 0.2 ml of serum

sample in a centrifuge tube. The mixture was shaken and the tube was allowed to stand for 5 minutes before being centrifuged for 5-10 minutes at 1000 rpm. In this process the sample had already been diluted 10 times, therefore, only 0.2 ml of the clear supernatant was added into the mixture of 2.8 ml BUN acid reagent and 2 ml BUN color reagent, and was mixed thoroughly before followed the steps as described for standard solution. From the value of the absorbances the concentrations of urea in serum samples were calculated from the calibration curve.

2.11.4 Comparison of the results

The conductometric urea biosensor was validated by comparing the results to those of the Fearon and the Berthelot reactions. 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 (Triola, 1998) were used in this work.

The regression line (Figure 10) 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 10 (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 10 (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. Such tests are performed by determining the errors in the slope (S_m) and intercept (S_C) of the regression line at 95% significant level. If $m \pm S_m$ cover 1 and $C \pm S_c$ cover zero there are no systematic errors and the results are then accepted (Miller and Miller, 1993).

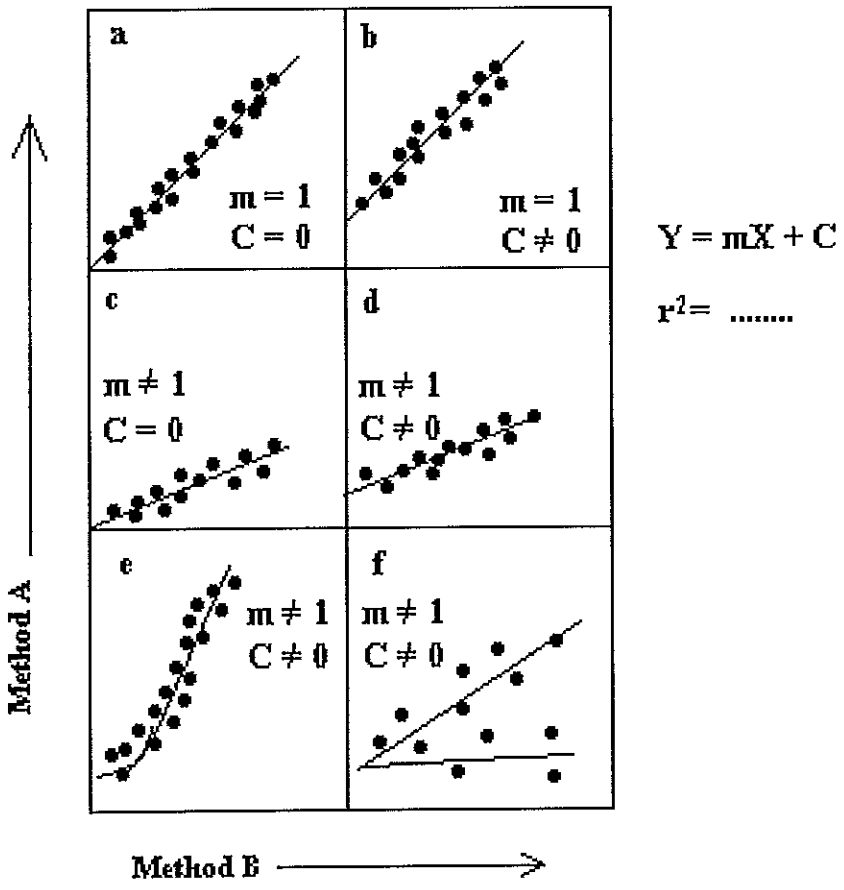

Figure 10 The use of a regression line to compare two analytical methods; (a) shows perfect agreement between the two methods for all the samples; (b)-(f) illustrate the result of various type of systematic errors of the slope and/or the interception.
(Redrawn form Miller and Miller, 1993)

The Wilcoxon signed rank test is one type of statistical tests uses to handle data which may not be normally distributes. 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 lower of these two figures was taken as the test statistic. If this value is less than the one given in Table 1 the two populations varied significantly (P = 0.05).

Table 1. Critical values for the Wilcoxon signed rank test: statistic at P = 0.05 for n = 6 to 37 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	Two-tailed test	n	Two-tailed test
6	0	22	65
7	2	23	70
8	3	24	75
9	5	25	81
10	8	26	86
11	10	27	92
12	13	28	98
13	17	29	105
14	21	30	112
15	25	31	119
16	29	32	130
17	33	33	143
18	37	34	154
19	41	35	167
20	55	36	180
21	60	37	199

Chapter 3

Results and Discussion

3.1 Characteristics of the flow biosensor response

The typical conductivity response of the urea-sensor is shown in Figure 11. The amplitude of the signal (H) which directly relate to urea concentration was measured. The response time, wash out time and analysis time were also considered.

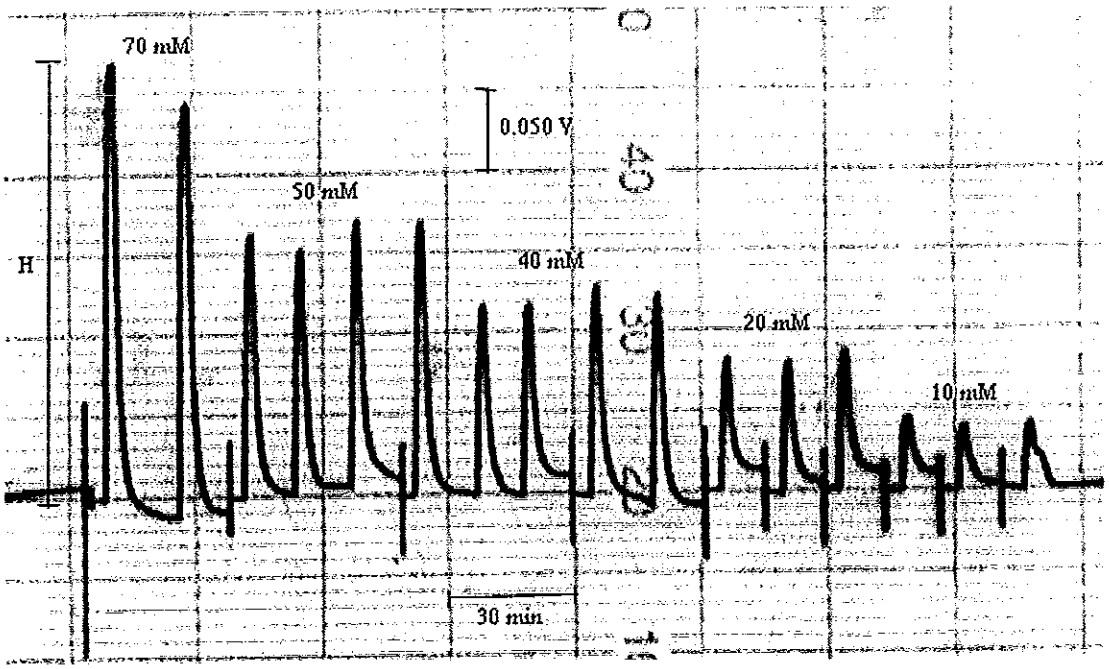

Figure 11. Response of immobilized urease to urea measured as the change in conductivity of the solution and recorded as a voltage signal by the analytical system. This figure shows the responses when one-minute pulses of urea solutions were passed through the flow-through system with a small dialyser at 0.5 ml min^{-1} for the buffer line and 0.5 ml min^{-1} for sample line.

3.2 Optimization of the flow systems

The flow rates and the sample volume of the flow-through and the flow-injection systems were optimized to obtain a maximum signal in the shortest period of time.

3.2.1 The flow-through system

3.2.1.1 Flow rates

a) Flow rate of the buffer line

The flow rate of the buffer line would effect the dispersion behavior, the retention time of the analyte in the enzyme reactor and in the conductivity cell. A slow flow rate allowed a sample to retain longer in the enzyme reactor, so the reaction was more completed. The retention time of the analyte in the conductivity cell would also be longer, hence the signal was higher for a slower flow rate. Table 2 and Figure 12 show the responses at different flow rates. The peak heights and sensitivities increased as the flow rate decreased and they differ significantly (P= 0.05) between each flow rate. However, the slower the flow rate the longer the analysis time. Therefore, several factors must be considered. In this case the flow rate of 0.50 ml min^{-1} was chosen because the sensitivity was only 8% lower than 0.25 ml min^{-1}, but the analysis time was much shorter (15 min compared to 22 min).

Table 2 Responses of a flow-through urea sensor system at different flow rates of the buffer line. (ND = non-detectable)

Concentration	Conductivity change (V) at flow rate					
(mM)	0.25 ml min^{-1}	0.40 ml min^{-1}	**0.50 ml min^{-1}**	0.60 ml min^{-1}	0.75 ml min^{-1}	1.00 ml min^{-1}
10	0.070	0.050	**0.050**	0.050	0.040	ND
20	0.100	0.080	**0.070**	0.070	0.050	0.030
30	0.130	0.100	**0.090**	0.090	0.070	0.050
40	0.170	0.140	**0.140**	0.130	0.110	0.080
50	0.230	0.190	**0.180**	0.160	0.130	0.100
60	0.260	0.210	**0.200**	0.180	0.150	0.130
70	0.290	0.240	**0.230**	0.220	0.190	0.160
80	0.330	0.290	**0.270**	0.250	0.220	0.190
90	0.350	0.330	**0.320**	0.300	0.270	0.220
Analysis time (min)	**18-22**	**14-18**	**10-15**	**10-13**	**7-10**	**6-8**
Slope	**0.0037**	**0.0035**	**0.0034**	**0.0031**	**0.0029**	**0.0027**
r^2	**0.9894**	**0.9921**	**0.9891**	**0.9888**	**0.9786**	**0.9956**

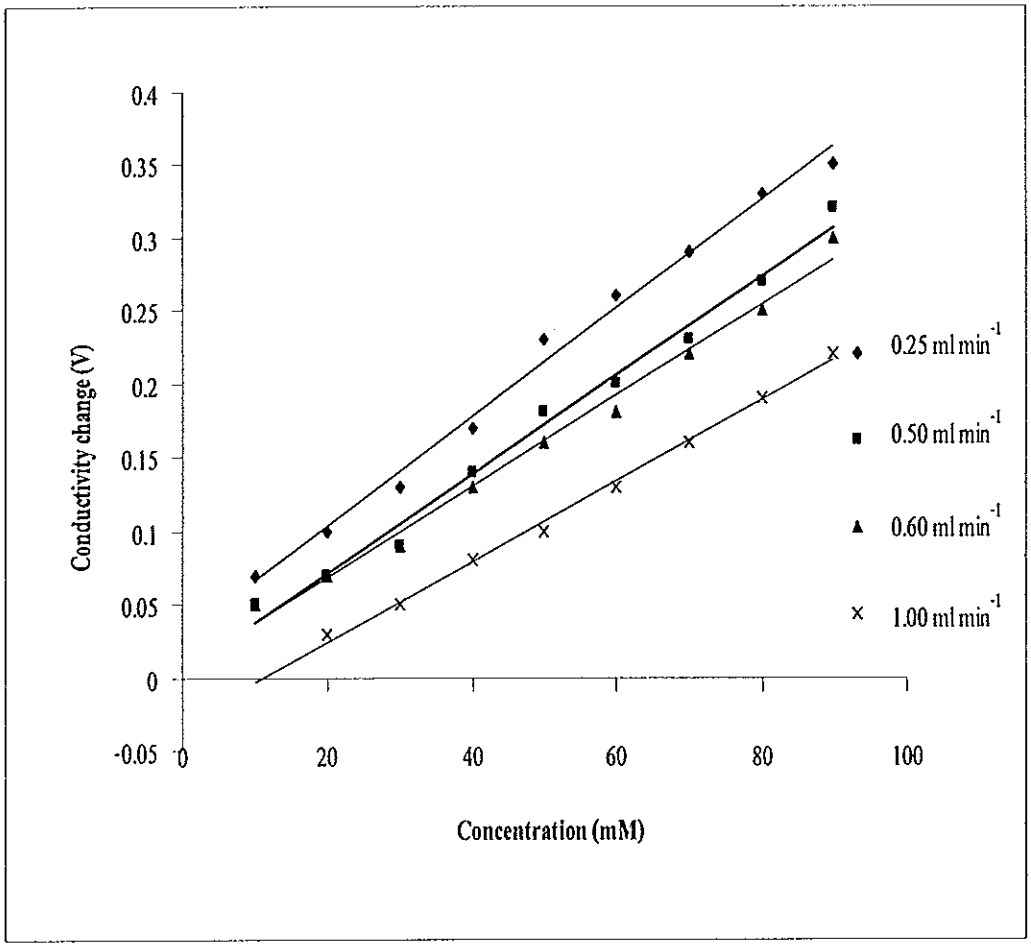

Figure 12 Responses of a flow-through urea sensor system at different flow rates of the buffer line.

b) Flow rate of the sample line

Sample line's flow rate directly affected the diffusion efficiency of urea through the dialysis membrane. The slow flow rate allows longer interaction between the analyte and diffusion area of the dialyser, hence the diffusion efficiency is increased. Because of this, a lower flow rate would give a higher signal. However, the slow flow rate also allows the increase of the dispersion phenomenal in the direction of flow and the analyte has more time to be diluted along the flow line. Table 3 and Figure 13 show the effect of the flow rates of the sample line on the responses of the system. At flow rates 1.00, 0.75, 0.50 and 0.25 ml min^{-1}, peak height increased with decreasing flow rates and differed significantly from each other. However, at a lower flow rate (0.10 ml min^{-1}) the amplitude of the signals did not increase significantly (P=0.05) and this may cause by the increase of the dispersion effect. It can be seen that the highest signal, highest sensitivity with the shortest analysis time is at flow rate 0.25 ml min^{-1} and this was chosen as an optimum.

Table 3 Responses of a flow-through urea sensor system at different flow rates of the sample line. (ND = non detectable)

Concentratio (mM)	Conductivity change (V) at flow				
	0.10 ml $^{-1}$	**0.25 ml $^{-1}$**	0.50 ml $^{-1}$	0.75 ml $^{-1}$	1.00 ml $^{-1}$
10	0.060	**0.060**	0.050	0.040	ND
20	0.100	**0.080**	0.070	0.060	0.050
30	0.120	**0.110**	0.090	0.080	0.070
40	0.150	**0.150**	0.140	0.120	0.100
50	0.190	**0.200**	0.180	0.160	0.140
60	0.250	**0.240**	0.200	0.190	0.170
70	0.290	**0.280**	0.230	0.210	0.200
80	0.320	**0.310**	0.270	0.250	0.230
90	0.370	**0.360**	0.320	0.300	0.270
Analysis time	**19-22**	**14-16**	**12-15**	**10-14**	**10-14**
Slope	**0.0039**	**0.0039**	**0.0034**	**0.0032**	**0.0033**
r^2	**0.9896**	**0.9936**	**0.9891**	**0.9894**	**0.9965**

Table 3 Responses of a flow-through urea sensor system at different flow rates of the sample line. (ND = non detectable)

Concentratio (mM)	Conductivity change (V) at flow				
	0.10 ml $^{-1}$	**0.25 ml $^{-1}$**	0.50 ml $^{-1}$	0.75 ml $^{-1}$	1.00 ml $^{-1}$
10	0.060	**0.060**	0.050	0.040	ND
20	0.100	**0.080**	0.070	0.060	0.050
30	0.120	**0.110**	0.090	0.080	0.070
40	0.150	**0.150**	0.140	0.120	0.100
50	0.190	**0.200**	0.180	0.160	0.140
60	0.250	**0.240**	0.200	0.190	0.170
70	0.290	**0.280**	0.230	0.210	0.200
80	0.320	**0.310**	0.270	0.250	0.230
90	0.370	**0.360**	0.320	0.300	0.270
Analysis time	**19-22**	**14-16**	**12-15**	**10-14**	**10-14**
Slope	**0.0039**	**0.0039**	**0.0034**	**0.0032**	**0.0033**
r^2	**0.9896**	**0.9936**	**0.9891**	**0.9894**	**0.9965**

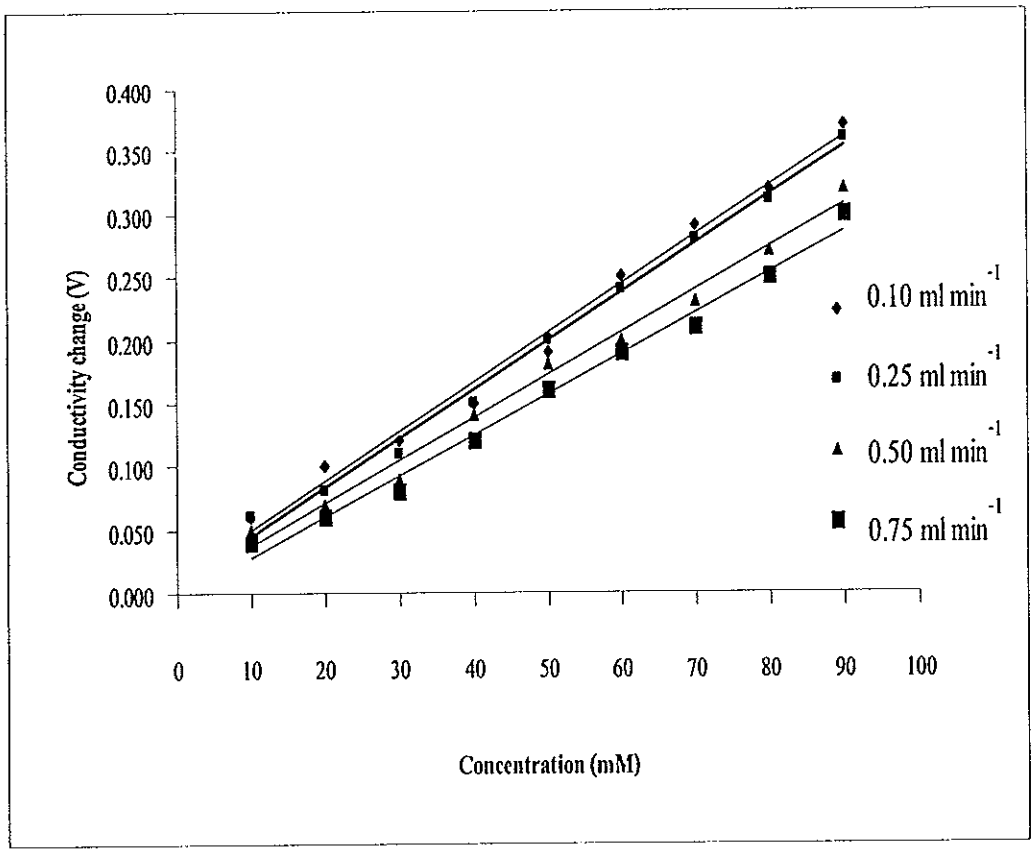

Figure 13 Responses of a flow-through urea sensor system at different flow rates of the sample line.

3.2.1.2 Sample volume

To optimize the sample volume, 0.125, 0.250, 0.500 and 0.725 of 10, 20, 30, 40, 50, 60, 70, 80 and 90 mM of urea standard solutions were pulsed into the system. The results were shown in Table 4 and Figure 14. The responses increased as the sample volume increased. However, when the sample volume was more than 0.25 ml the responses were not significant increased (P =0.05). This may be because the rate of the reaction depended not only on the amount of the substrate but also on the amount of the enzyme. In this case there may be too much substrate for the same amount of enzyme, thus, the response could not be improved. Moreover, the analysis time also increased, since the dispersion increased with increasing sample volume. So, The sample volume 0.25 ml was chosen for this system.

Table 4 Responses of a flow-through urea sensor system at different sample volume.

Concentration	Conductivity change (V) at sample volume			
(mM)	0.125 ml	**0.250 ml**	0.500 ml	0.725 ml
10	ND	**0.050**	0.060	0.060
20	0.060	**0.080**	0.080	0.080
30	0.090	**0.110**	0.100	0.110
40	0.130	**0.140**	0.150	0.150
50	0.170	**0.190**	0.200	0.200
60	0.193	**0.240**	0.250	0.240
70	0.233	**0.280**	0.290	0.280
80	0.254	**0.310**	0.310	0.310
90	0.310	**0.350**	0.360	0.360
Analysis time (min)	**10-14**	**11-16**	**16-20**	**19-22**
Slope	**0.0035**	**0.0039**	**0.004**	**0.0039**
r^2	**0.9936**	**0.9941**	**0.9875**	**0.9936**

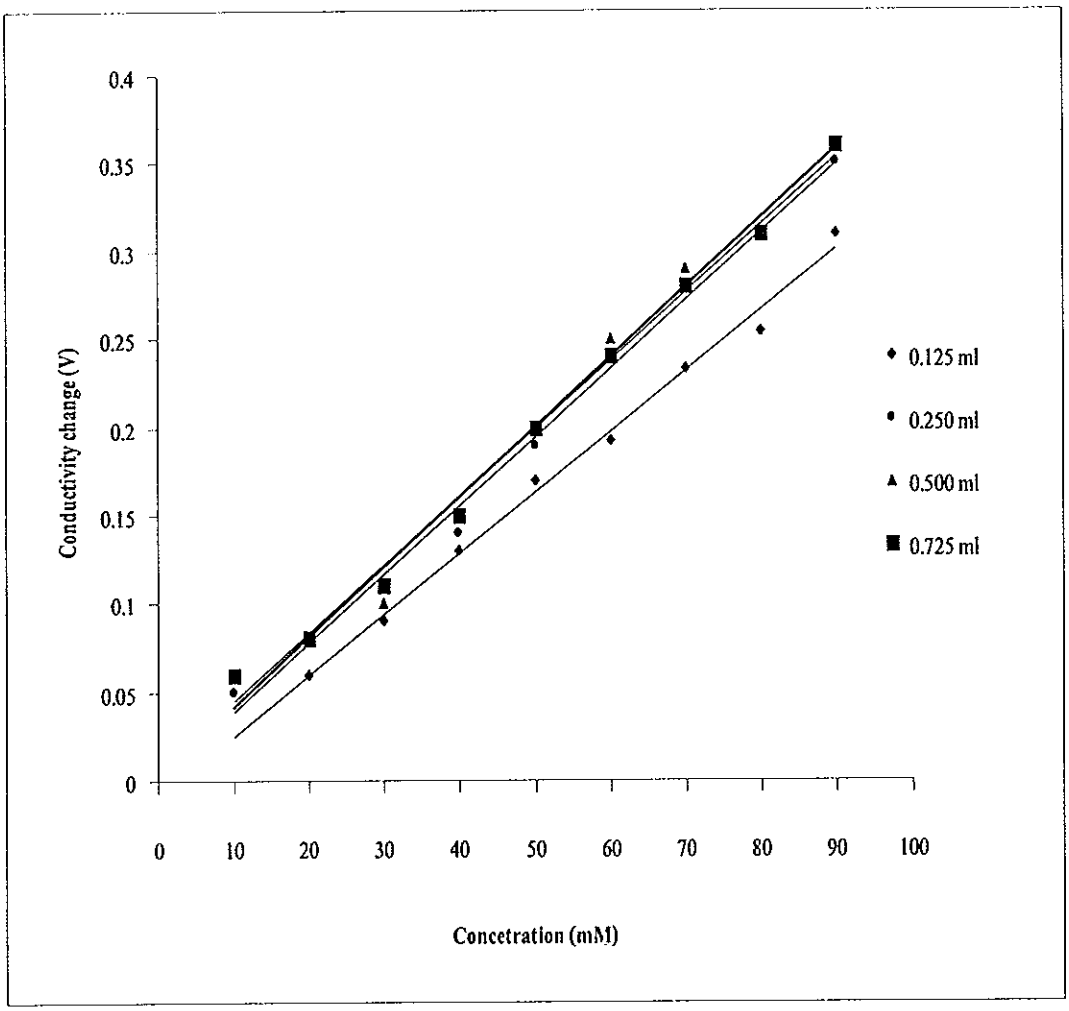

Figure 14 Responses of a flow-through urea sensor system at different sample volume.

From the results, the optimum conditions for the flow-through urea conductometric biosensor are;

- flow rate of the buffer line 0.50 ml min^{-1}
- flow rate of the sample line 0.25 ml min^{-1}
- the sample volume 0.25 ml.

3.2.2 The flow-injection system

3.2.2.1 Flow rates

a) Flow rate of the buffer line

The effect of the flow rate of the buffer line on the sensor response was investigated by varied the flow rate from 0.20 to 0.75 ml min^{-1}. The responses of 10 to 90 mM urea at different flow rates are shown in Table 5 and Figure 15. At the flow rates 0.75, 0.50 and 0.25 ml min^{-1} the sensitivity and peak heights were significant increased (P = 0.05). Similar to the flow-through system when the flow rate was below 0.25 ml min^{-1}, the sensitivity and peak height could not be improved. In term of sensitivity, peak height and analytical time, flow rate 0.25 ml min^{-1} was then chosen as an optimum of flow rate of buffer line.

Table 5 Responses of a flow-injection urea sensor system at different flow rates of the buffer line.

Concentration (mM)	Conductivity change (V) at flow rate			
	0.20 ml min^{-1}	**0.25 ml min^{-1}**	0.50 ml min^{-1}	0.75 ml min^{-1}
10	0.080	**0.070**	0.050	0.030
30	0.180	**0.160**	0.130	0.100
50	0.270	**0.260**	0.220	0.190
70	0.370	**0.370**	0.340	0.280
90	0.460	**0.450**	0.400	0.370
Analysis time	**17-20**	**13-15**	**8-12**	**8-10**
Slope	**0.0048**	**0.0049**	**0.0046**	**0.0043**
r^2	**0.9997**	**0.9980**	**0.9920**	**0.9978**

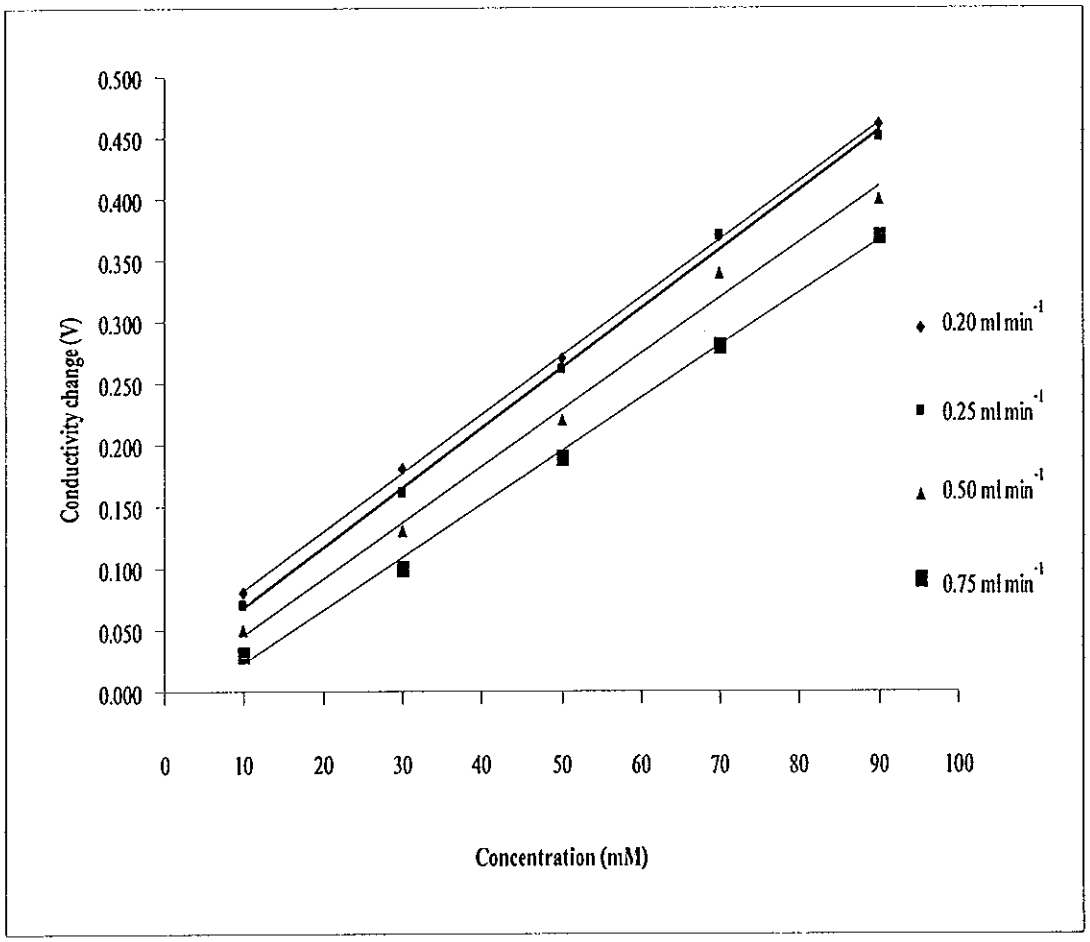

Figure 15 Responses of a flow-injection urea sensor system at different flow rates of the buffer line.

b) Flow rate of the sample line

Flow rates of 0.20, 0.25, 0.50 and 0.75 were investigated. The results are shown in Table 6 and Figure 16. The highest signals were obtained at 0.20 ml min^{-1}. However, the analysis time was much longer than others. At 0.25 ml min^{-1} the peak responses were nearly equal to those of 0.02 ml min^{-1} but the analysis time was shorter and the sensitivity was better so this was chosen to be used in further analysis.

Table 6 Responses of a flow-injection urea sensor system at different flow rates of the sample line.

Concentration (mM)	Conductivity change (V) at flow rate			
	0.20 ml min^{-1}	**0.25 ml min^{-1}**	0.50 ml min^{-1}	0.75 ml min^{-1}
10	0.070	**0.060**	0.050	0.030
30	0.180	**0.160**	0.120	0.100
50	0.270	**0.250**	0.200	0.180
70	0.390	**0.380**	0.310	0.260
90	0.430	**0.440**	0.380	0.350
Analysis time (min)	**14-18**	**13-15**	**10-13**	**10-13**
Slope	**0.0047**	**0.0049**	**0.0043**	**0.0040**
r^2	**0.9819**	**0.9913**	**0.9941**	**0.9981**

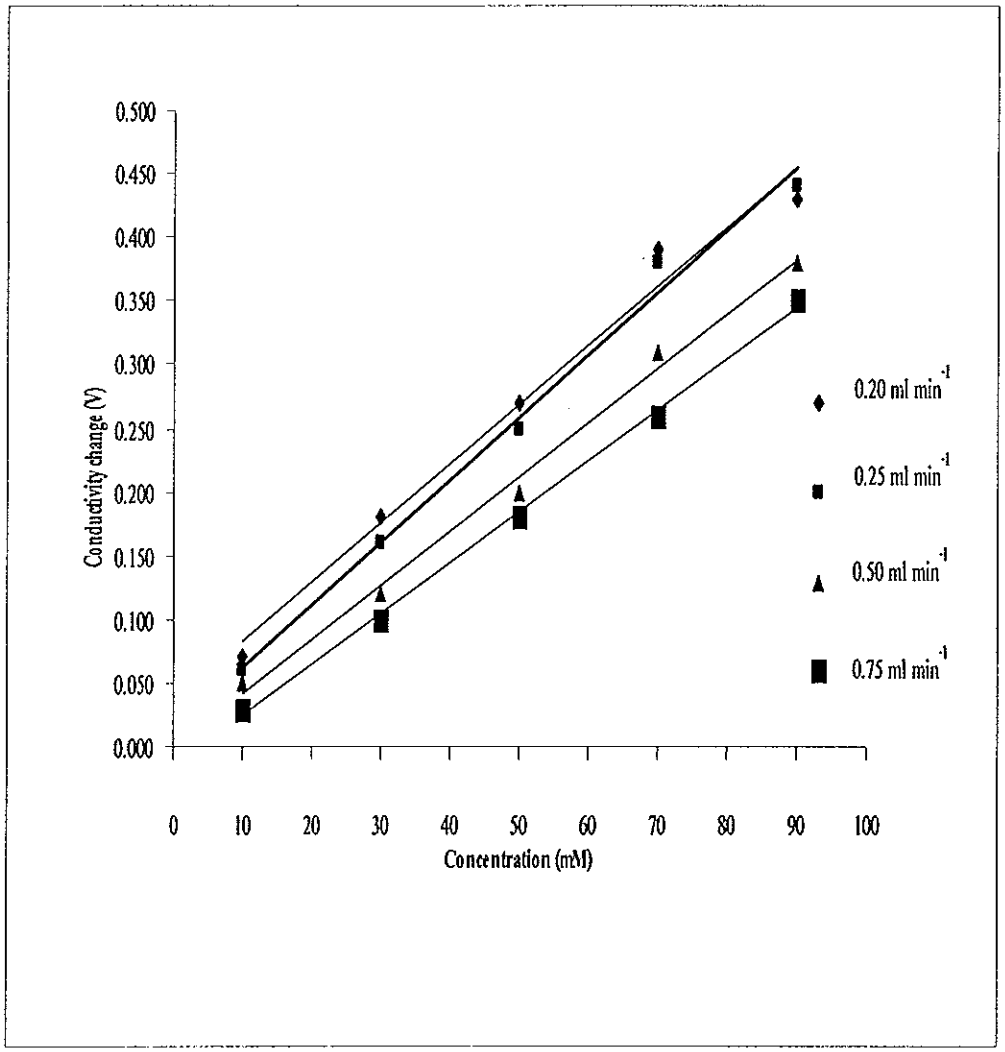

Figure 16 Responses of a flow-injection urea sensor system at different flow rates of the sample line.

3.2.2.2 Sample volume

Table 7 and Figure 17 show the effect of the sample size on the flow-injection biosensor. The responses increase significantly (P=0.05) with the sample volumes until 0.25 ml. Therefore, 0.25 ml of sample volume was chosen.

Table 7 Responses of a flow-injection urea sensor system at different sample volume.

Concentratio (mM)	Conductivity change (V) at sample volume				
	0.15 ml	0.20 ml	**0.25 ml**	0.40 ml	0.50 ml
10	0.030	0.050	**0.070**	0.070	0.080
30	0.120	0.160	**0.180**	0.200	0.210
50	0.200	0.230	**0.260**	0.270	0.290
70	0.310	0.350	**0.390**	0.400	0.430
90	0.380	0.420	**0.460**	0.470	0.480
Analysis time	**9-13**	**11-14**	**13-16**	**14-19**	**17-22**
Slope	**0.0045**	**0.0047**	**0.0050**	**0.0050**	**0.0051**
r^2	**0.9966**	**0.9932**	**0.9932**	**0.9893**	**0.9826**

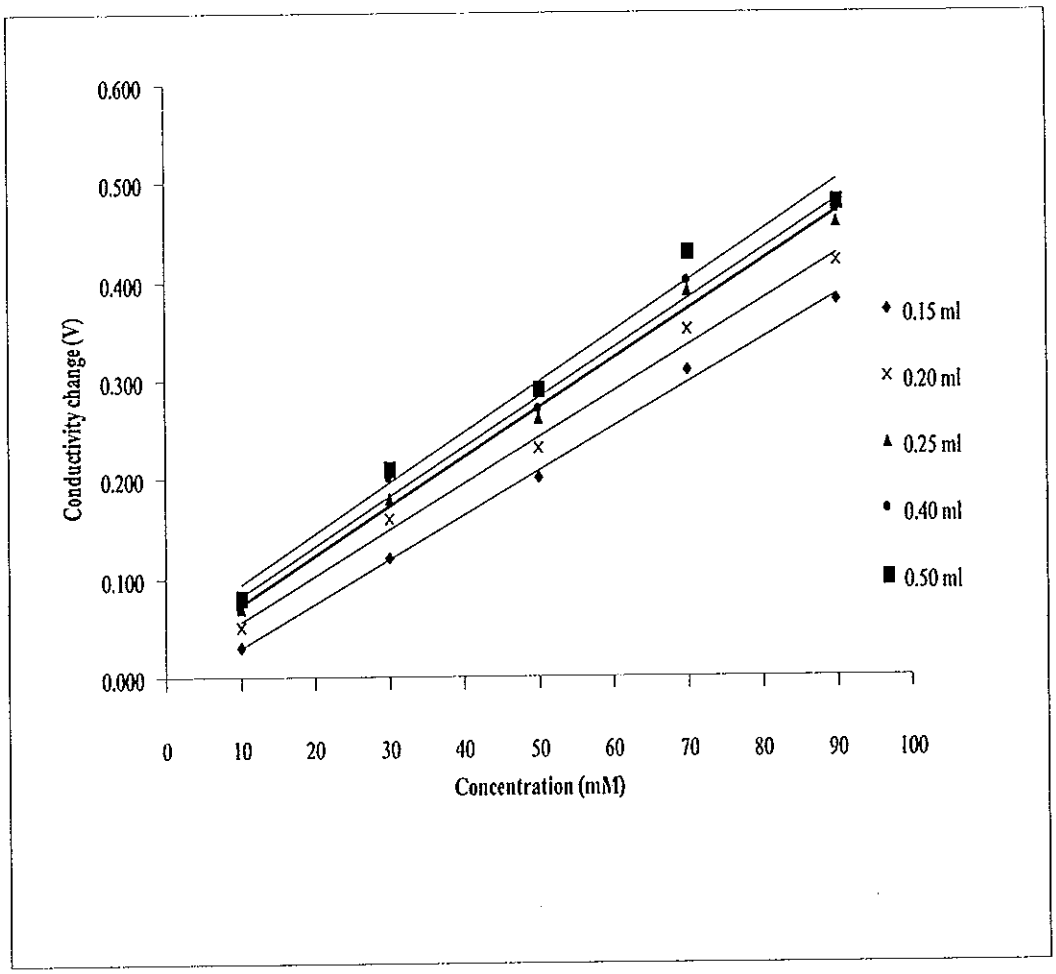

Figure 17 Responses of a flow-injection urea sensor system at different sample volume.

In summary, the optimum conditions for the flow-injection urea conductometric biosensor are;

- flow rate of the buffer line 0.25 ml min^{-1}
- flow rate of the sample lines 0.25 ml min^{-1}
- the sample volume 0.25 ml.

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

The same urea standard solutions (1, 10, 20, 30, 40, 50, 60, 70, 80 and 90) were tested by the two proposed systems at their optimum conditions (3.2.1 and 3.1.2 respectively). The comparison was considered using several analytical parameters; *i.e.* linear range, sensitivity, precision, limit of detection, and analysis time. Both systems, the flow-through and the flow-injection, have a wide linear range, 5-90 mM and 1-90 mM, with satisfactory regression coefficient (r^2) 0.9947 and 0.9978 respectively (Table 8 and Figure 18). Although analysis time for the flow-injection was longer than the flow-through system (15 min compared to 12 min), the sensitivity was 25% higher and the limit of detection was 5 times lower (1 mM compared to 5 mM). As for the precision, the flow-injection system provided a better %RSD for all concentrations. These were the reasons that the flow-injection system was chosen for this work.

Table 8 Comparison of the results obtained with the flow-through and the flow-injection systems at their optimum conditions. (ND = non-detectable)

Concentration (mM)	Conductivity change (V) with					
	Flow-injection			Flow-through		
	mean	SD	%RSD	mean	SD	%RSD
1	0.01	0.003	30.00	ND		
5	0.03	0.003	10.00	0.03	0.005	16.67
10	0.07	0.005	7.14	0.05	0.008	16.00
20	0.11	0.004	3.64	0.08	0.008	10.00
30	0.16	0.003	1.88	0.11	0.007	6.36
40	0.2	0.005	2.50	0.14	0.006	4.29
50	0.26	0.004	1.54	0.19	0.009	4.74
60	0.31	0.003	0.97	0.24	0.007	2.92
70	0.37	0.005	1.35	0.28	0.008	2.86
80	0.42	0.003	0.71	0.31	0.008	2.58
90	0.45	0.005	1.11	0.35	0.009	2.57
analysis time (min)	13-15			9-12		
Slope	0.0050			0.0038		
r^2	0.9978			0.9947		

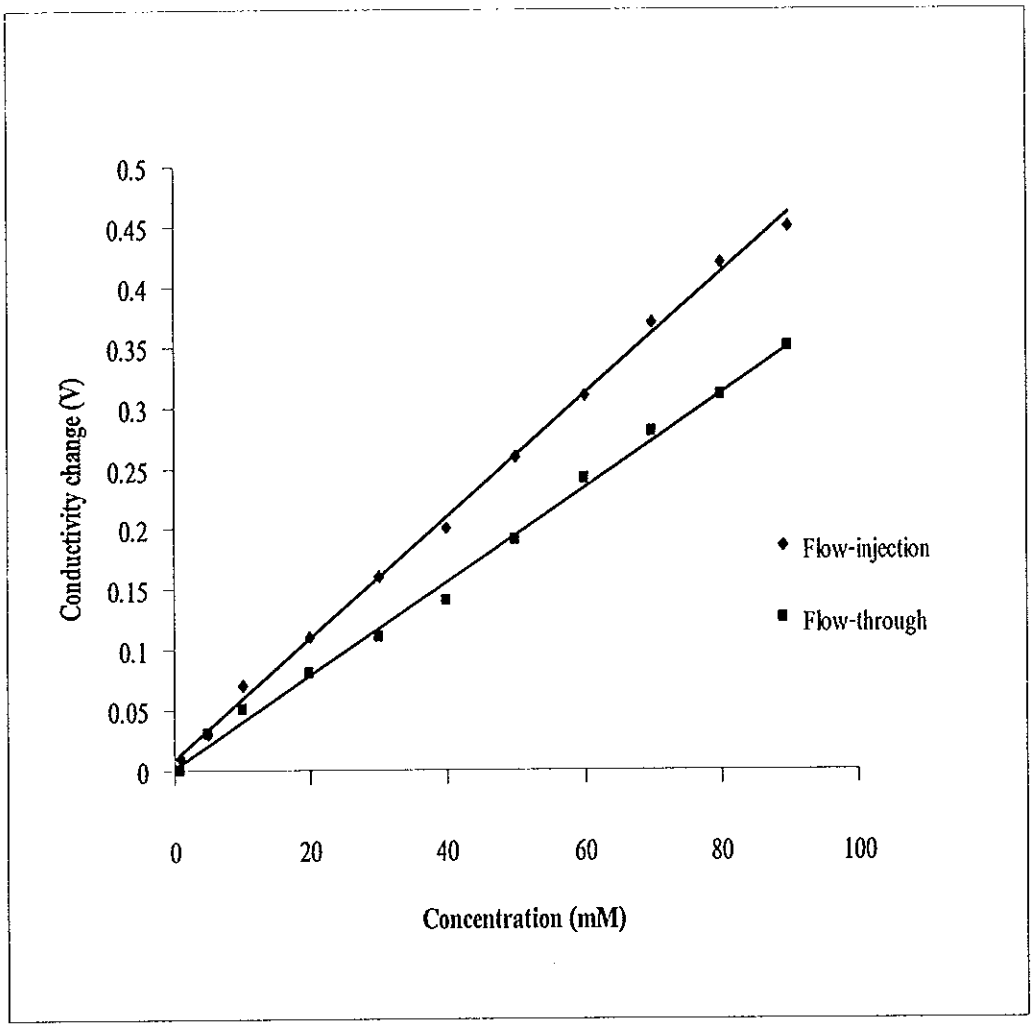

Figure 18 Comparison of the responses obtained with the flow-through and flow-injection systems at their optimum conditions.

3.2.4 Optimization of the flow-injection system using a large area dialyser

The results for the responses of different flow rates and sample volumes were shown in Tables 9-11 and Figures 19-21. The optimum flow rates and sample volume were the same as in the small dialyser system, i.e. 0.25 ml min^{-1} for both the buffer and sample line flow rates and the sample volume was 0.25 ml. The only different being the analysis time which was longer (23 min compared to 15 min). This was expected since the larger dialyser has a longer track and the urea from the sample would be able to move into a larger volume of the buffer on the other side of the membrane that passed through the enzyme reactor. Hence, a longer analysis time.

Table 9 Responses of a flow-injection urea sensor system with the large dialyser at different flow rates of the buffer. (ND = non-detectable)

Concentration (mM)	Conductivity change (V) at the flow rate			
	0.20 ml min^{-1}	0.25 ml min^{-1}	0.50 ml min^{-1}	0.75 ml min^{-1}
1	0.06	0.05	ND	ND
2	0.12	0.12	0.09	0.07
3	0.26	0.24	0.2	0.18
4	0.36	0.35	0.29	0.26
5	0.4	0.38	0.35	0.32
6	0.55	0.52	0.48	0.44
7	0.62	0.61	0.56	0.52
8	0.78	0.76	0.71	0.67
9	0.85	0.84	0.78	0.72
10	0.96	0.96	0.87	0.83
Analysis time (min)	20-30	18-25	17-23	17-22
Slope	0.1015	0.1015	0.0985	0.0947
r^2	0.9938	0.9934	0.9953	0.9942

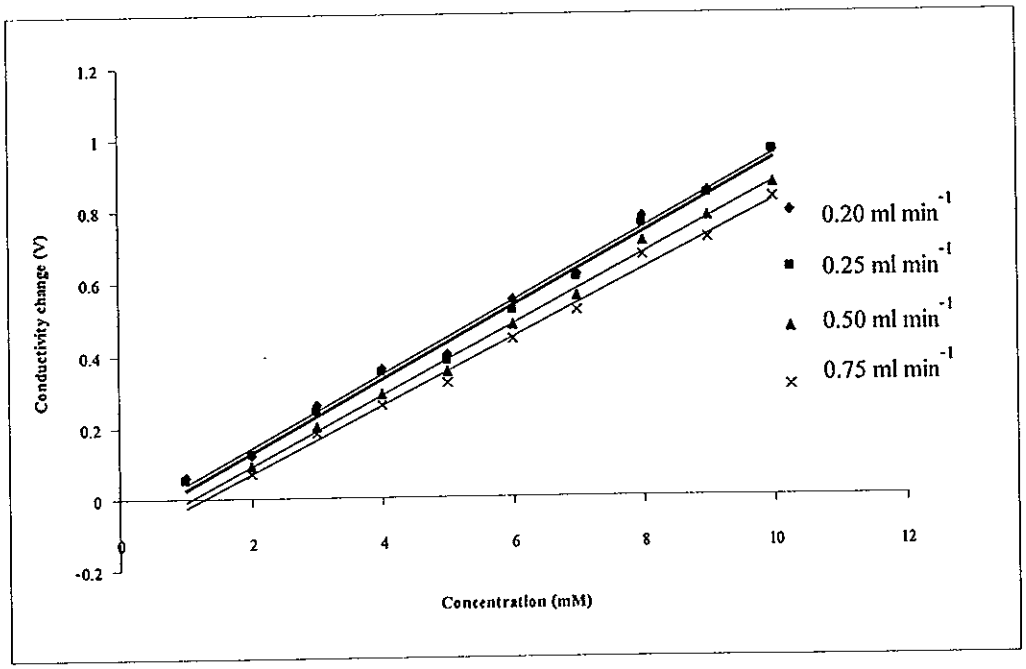

Figure 19 Responses of a flow-injection urea sensor system with the large dialyser at different flow rates of the buffer line.

Table 10 Responses of a flow-injection urea sensor system with the large dialyser at different flow rates of the sample. (ND = non-detectable)

Concentration (mM)	Conductivity change (V) at the flow rate			
	0.20 ml/min^{-1}	0.25 ml/min^{-1}	0.50 ml/min^{-1}	0.75 ml/min^{-1}
1	0.050	0.040	ND	ND
2	0.110	0.120	0.080	0.070
3	0.250	0.230	0.210	0.190
4	0.370	0.360	0.270	0.270
5	0.410	0.390	0.360	0.310
6	0.560	0.510	0.460	0.410
7	0.630	0.600	0.570	0.530
8	0.770	0.770	0.700	0.670
9	0.840	0.840	0.780	0.710
10	0.960	0.960	0.880	0.820
Analysis time (min)	20-26	20-25	19-24	19-24
Slope	0.102	0.1022	0.0997	0.093
r^2	0.9952	0.9929	0.9962	0.9897

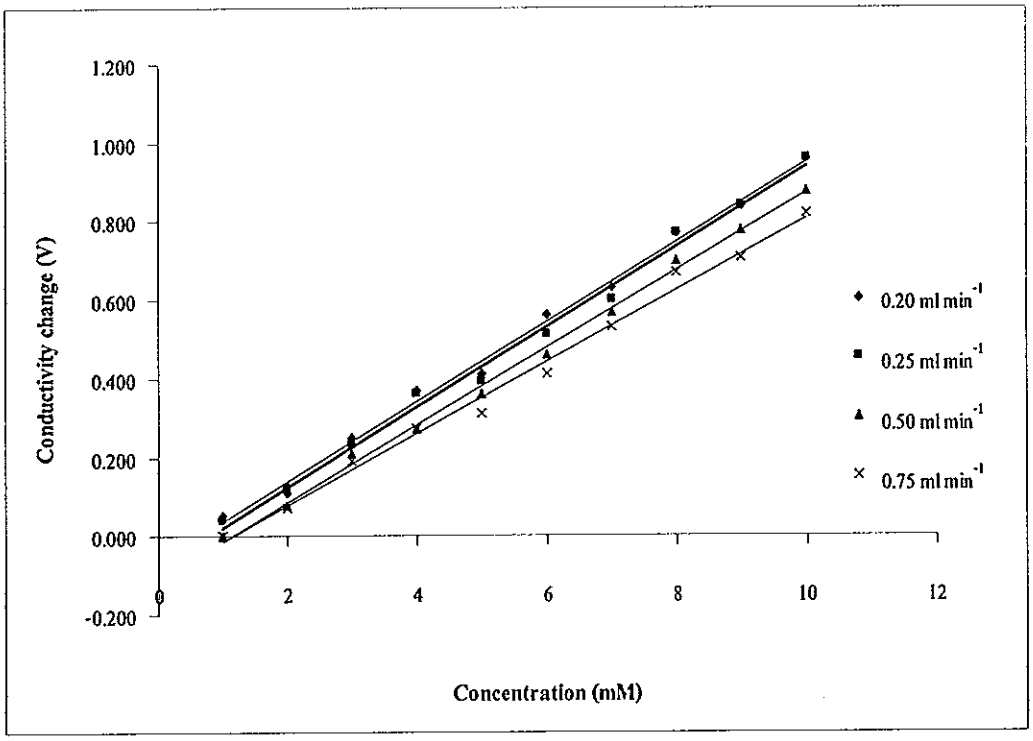

Figure 20 Responses of a flow-injection urea sensor system with the large dialyser at different flow rates of the sample.

Table 11 Responses of a flow-injection urea sensor system with the large dialyser at different sample volume. (ND = non-detectable)

Concentration (mM)	Conductivity change (V) at sample volume			
	0.15 ml	0.20 ml	0.25 ml	0.40 ml
1	ND	0.050	0.050	0.060
2	0.080	0.100	0.130	0.140
3	0.150	0.200	0.240	0.260
4	0.220	0.280	0.350	0.360
5	0.350	0.380	0.420	0.430
6	0.400	0.460	0.530	0.550
7	0.470	0.550	0.620	0.640
8	0.550	0.640	0.790	0.790
9	0.610	0.720	0.850	0.860
10	0.690	0.800	0.970	0.970
Analysis time (min)	15-19	19-22	19-23	22-27
Slope	0.0767	0.0859	0.1030	0.1021
r^2	0.9943	0.9986	0.9958	0.9973

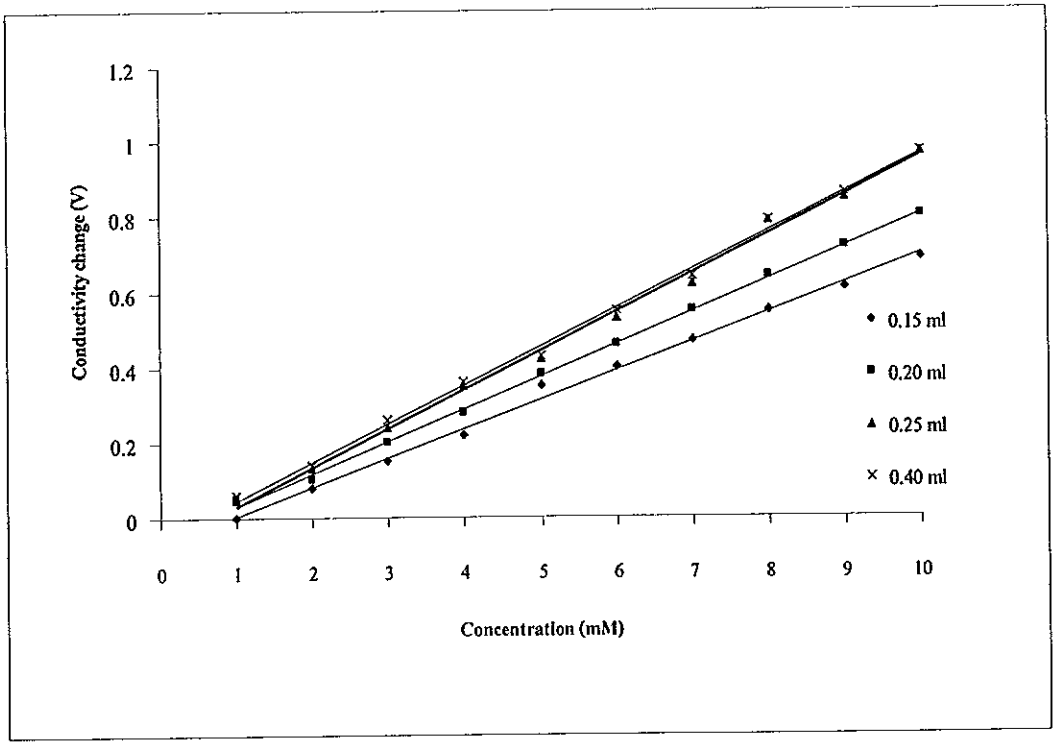

Figure 21 Responses of a flow-injection urea sensor system with the large dialyser at different sample volume.

3.2.5 Comparison of the small and large dialysers

The large (1.5 x 298 mm^2) and the small (1.5 x 49 mm^2) dialyser systems were tested under their optimum conditions. The results and the calibration curves were shown in Tables 12-13 and Figures 22-23. The differences being peak height, precision, sensitivity, limit of detection, linear range and analysis time. The large dialyser system gave a 20 times higher sensitivity (0.1015 *c.f.* 0.0051 V/mM), approximately 14 times higher peak height and 2 times lower limit of detection (0.5 *c.f.* 1.0 mM) than the small one. This is mainly because of the amount of urea passing through the membrane into the reaction part increased with the diffusion area. For the precision, the large dialyser provided a better %RSD for all concentrations. However, the small dialyser system offered a wider linear range from 1 up to 90 mM, while the linear range of the large one was only form 0.5 to 10.0 mM. Shorter analysis time (15 min or less) was also a great advantage of the small dialyser system, whereby in the large dialyser system the time of a single measurement varied form 20 to 30 min, depending on the urea concentrations.

By considering the linear range and the analysis time, the small diffusion area of dialyser may be suitable for studying the factors that may affect the system. On the other hand, to measure urea in serum, the samples were diluted 10 times and the concentrations of urea in the diluted samples would fall into the linear range of the large dialyser. For some samples with low content of urea, after dilution the concentration may only be 0.5-0.6 mM. This is lower than the detection limit of the small dialyser system but should be detected by the large dialyser system where the detection limit was 0.5 mM. So, the system with the large dialyser was chosen for blood urea analysis.

Table 12 The conductivity change of the flow-injection system with the small dialyser. (ND = non-detectable)

Urea concentration (mM)	Conductivity change (V)					
	First	Second	Third	mean	SD	%RSD
0.5	ND	ND	ND	ND	ND	ND
1	0.010	0.015	0.014	0.013	0.003	20.35
5	0.025	0.032	0.035	0.031	0.005	16.73
10	0.065	0.072	0.073	0.070	0.004	6.23
20	0.114	0.108	0.110	0.111	0.003	2.76
30	0.158	0.162	0.165	0.162	0.004	2.17
40	0.195	0.203	0.199	0.199	0.004	2.01
50	0.260	0.255	0.259	0.258	0.003	1.03
60	0.310	0.317	0.308	0.312	0.005	1.52
70	0.372	0.375	0.366	0.371	0.005	1.24
80	0.420	0.423	0.417	0.420	0.003	0.71
90	0.452	0.447	0.452	0.450	0.003	0.64
analysistime (min)				15		
Slope				0.0051		
r^2				0.9976		

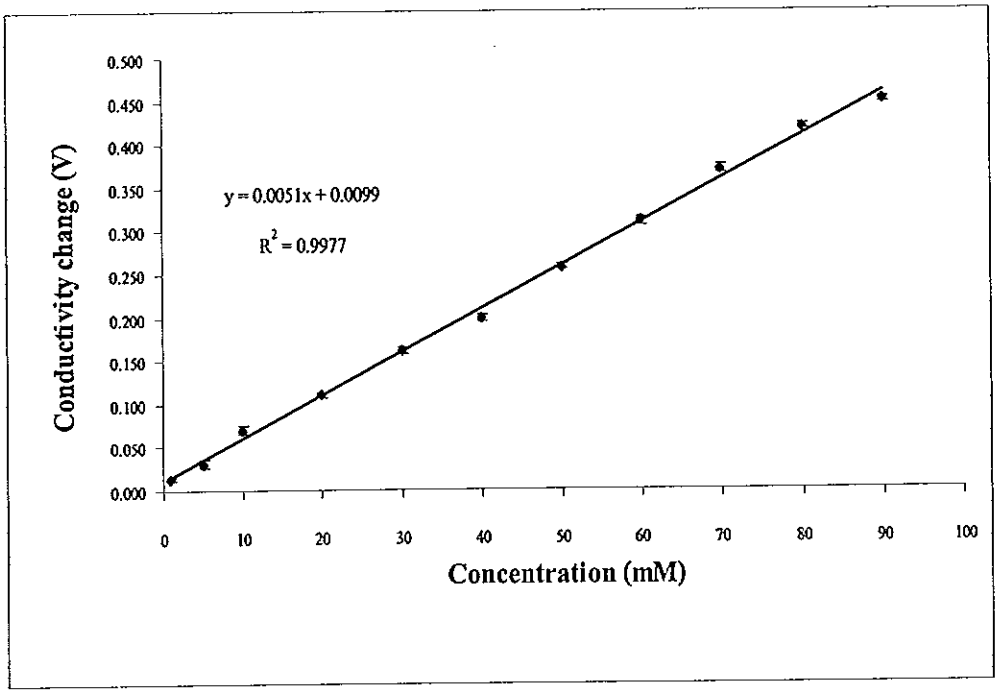

Figure 22 Calibration curve of urea using the flow-injection system with the small dialyser.

Table 13 The conductivity change of the flow-injection system with the large dialyser.

Urea concentration (mM)	Peak height (V)					
	First	Secound	Third	mean	SD	%RSD
0.5	0.015	0.020	0.024	0.020	0.005	22.93
1	0.055	0.048	0.047	0.050	0.004	8.72
2	0.133	0.125	0.133	0.130	0.005	3.54
3	0.235	0.243	0.250	0.243	0.008	3.09
4	0.351	0.344	0.359	0.351	0.008	2.14
5	0.415	0.425	0.418	0.419	0.005	1.22
6	0.536	0.523	0.532	0.530	0.007	1.26
7	0.626	0.620	0.618	0.621	0.004	0.67
8	0.782	0.790	0.800	0.791	0.009	1.14
9	0.852	0.844	0.860	0.852	0.008	0.94
10	0.980	0.967	0.973	0.973	0.007	0.67
analysis time (min)				25		
Slope				0.1015		
r^2				0.9955		

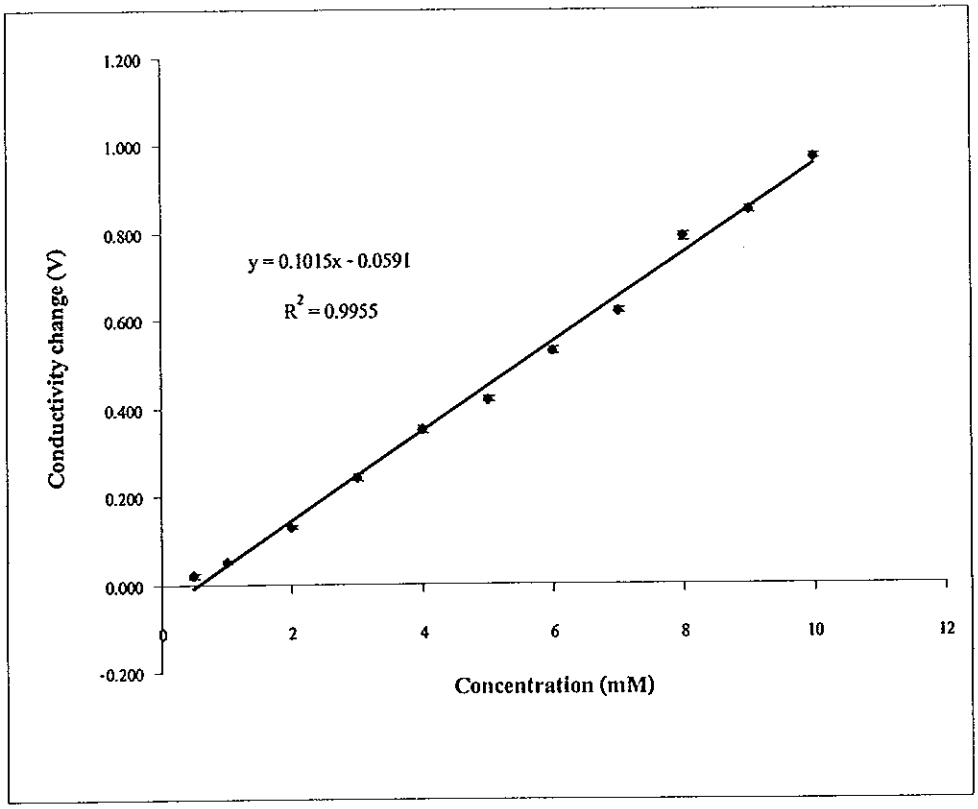

Figure 23 Calibration curve of urea using the flow-injection system with the large dialyser.

3.3 Lifetime of urea standard solution

The long-term storage stability of standard urea solutions (5 - 60 mM) were tested over a 7 day period by monitoring its calibration curve every day at two storage conditions, at room temperature (23° - 30°C) and 4°C. It was found for both conditions (Tables 14, 15 and Figures 24, 25) that the slopes of the calibration curve fluctuated around 0.0035 V/mM during a 7-day period. The linear range did not change considerably throughout this period and the linear regression coefficients were around 0.99. Therefore, it can be concluded that the lifetime of the urea standard solutions in glycine-NaOH buffer pH 8.8 + 0.02% sodium azide is at least 7 days for both storage conditions.

Table 14 Responses to urea standard solutions stored at room temperature (23° - 30° C) during a 7-day period.

Concentration (mM)	Conductivity change (V)						
	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
5	0.04	0.05	0.05	0.04	0.04	0.05	0.04
10	0.06	0.07	0.07	0.07	0.06	0.06	0.06
20	0.09	0.09	0.1	0.1	0.09	0.11	0.1
30	0.13	0.12	0.12	0.13	0.13	0.12	0.12
40	0.15	0.16	0.15	0.15	0.14	0.15	0.16
60	0.25	0.23	0.24	0.23	0.25	0.23	0.24
Slope	0.0037	0.0032	0.0033	0.0033	0.0036	0.0032	0.0035
r^2	0.9931	0.9960	0.9809	0.9944	0.9858	0.9903	0.9968

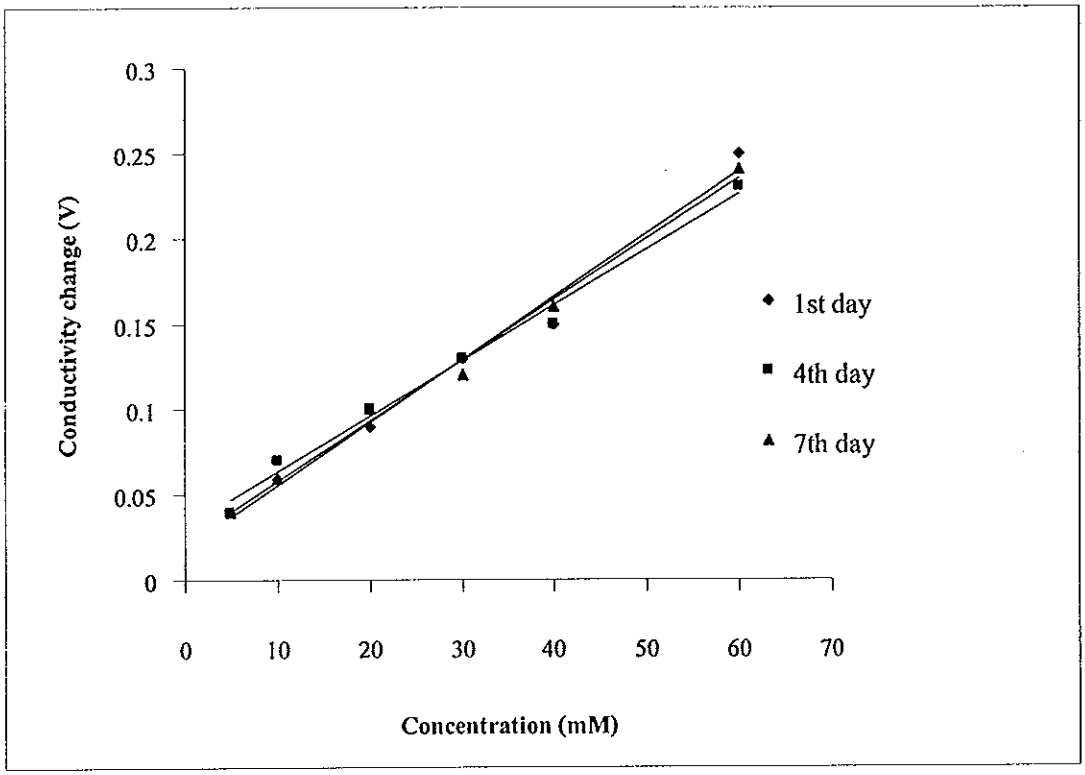

Figure 24 Responses to urea standard solutions stored at room temperature (23°-30°C) during a 7-day period.

Table 15 Responses to urea standard solutions stored at 4°C during a 7- day period.

Concentration (mM)	Conductivity change (V)						
	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
5	0.04	0.04	0.05	0.05	0.04	0.05	0.03
10	0.07	0.06	0.07	0.07	0.06	0.06	0.06
20	0.1	0.1	0.11	0.1	0.09	0.11	0.09
30	0.12	0.11	0.13	0.13	0.12	0.12	0.12
40	0.16	0.15	0.15	0.15	0.16	0.15	0.15
60	0.25	0.24	0.24	0.23	0.22	0.23	0.23
Slope	0.0036	0.0035	0.0033	0.0031	0.0033	0.0032	0.0035
r^2	0.9923	0.9892	0.9904	0.9958	0.9992	0.9903	0.9970

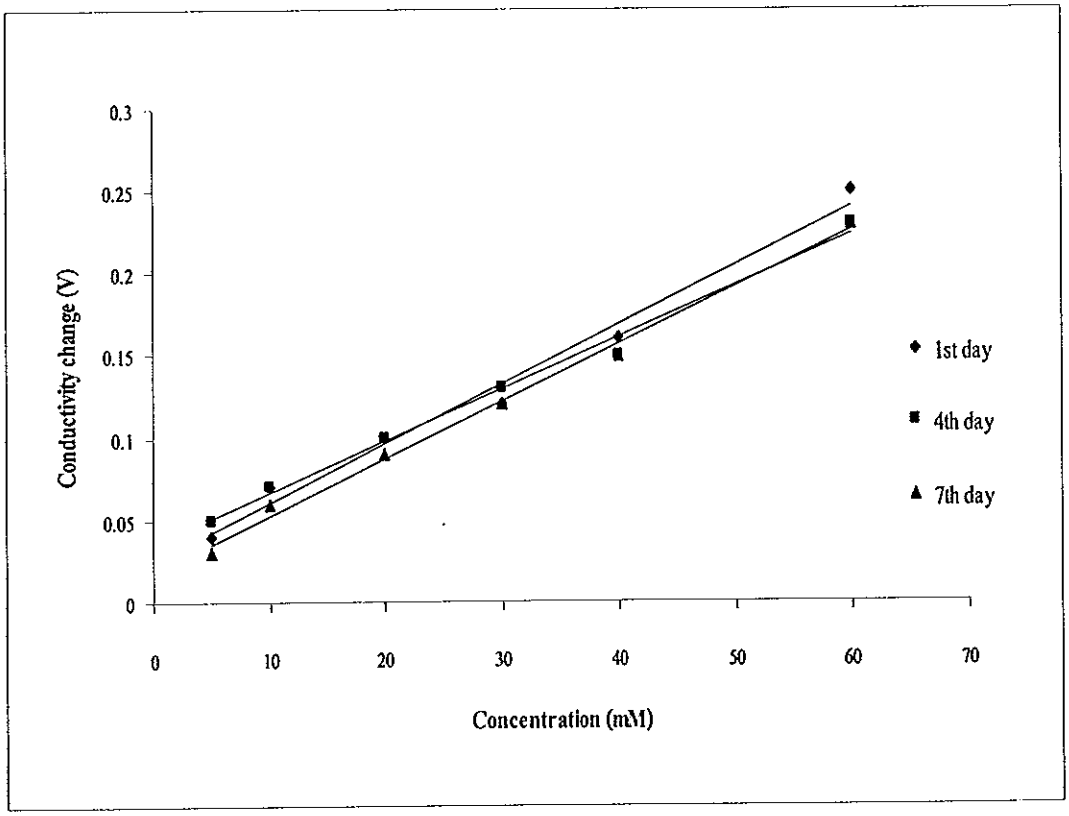

Figure 25 Responses to urea standard solutions stored at 4°C during a 7- day period.

3.4 Buffer concentration

Conductometric methods are relatively nonselective because all charge-carrying species are detected simultaneously. Buffer of low ionic strength must be used for the detection of low levels of substrate since detection limits are ultimately controlled by the ratio ΔG/G, where G is the conductance of the medium and ΔG is the conductance change that result from the enzyme process (Mikkelsen and Rechnitz, 1989). Therefore, the effect of buffer concentration on urea conductometric biosensor has to be examined because the sensitivity in the conductometric detection is ultimately controlled by the conductance change that resulted from the enzymatic reaction.

Table 16 and Figure 26 show the results obtained by using glycine-NaOH buffer pH 8.8 at 0.005, 0.010, 0.050 and 0.100 mM. In general the signal decreased as the buffer capacity increased. This caused the sensitivity and analysis time to decrease and the limit of detection to increase with the concentrations of buffer. That is at 0.005 mM the responses gave the largest amplitude, the best sensitivity (0.0056 V/mM) and the lowest limit of detection (0.5 mM). However, the analysis time was the longest and this can be attributed to local pH changes within the enzyme column due to the low capacity of buffer (Mikkenlsen and Rechnitz, 1989). From these results it seems that the lower the concentration the better. However, another matter to be considered is whether the buffer capacity would be enough when uses with serum sample. From our test with real samples the lowest buffer concentration that provided enough buffering capacity for serum was 0.05 M. Therefore, 0.05 M was chosen.

Table 16 Responses of a flow-injection urea sensor system at different buffer concentrations. (ND = non-detectable)

Conentration (mM)	Conductivity change (V) at the buffer concentration			
	0.005 M	0.010 M	0.050 M	0.100 M
0.5	0.010	ND	ND	ND
1	0.020	0.010	0.010	ND
5	0.040	0.030	0.030	0.020
10	0.080	0.070	0.060	0.040
20	0.130	0.110	0.110	0.080
25	0.160	0.140	0.130	0.100
50	0.290	0.260	0.250	0.220
Analysis time (min)	20	17	15	13
Slope	0.0055	0.0050	0.0048	0.0045
r^2	0.9990	0.9994	0.9997	1.0000

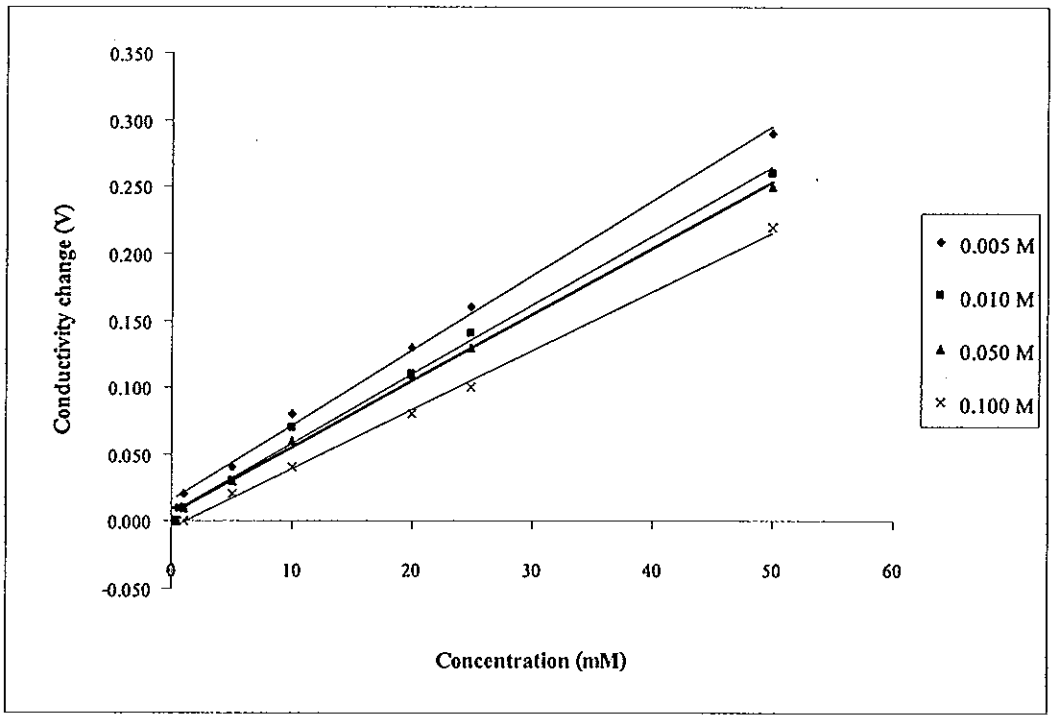

Figure 26 Responses of a flow-injection urea sensor system at different buffer concentrations.

3.5 Stability of immobilized enzyme

Generally after prolonged use of the enzyme, denaturation or inhibition of the enzyme may effect the response (Lehninger, 1987). The long-term performance of the enzyme reactor was evaluated intermittently for 6 month (263 h operation time). The results (Table 17 and Figure 27) show that a wide linear range (up to 90 mM) and good sensitivity (slope = 0.0038 V/mM) can still be obtained after 263 h operation time.

The first three conditions were different form the others, since they were the conditions used during the optimization steps. These were included to show the trend of the sensitivity and the peak height and to show that they did not change significantly during the 263 h period. Especially the last three conditions, though the sample volume was smaller the sensitivity was still the same, which indicated that the enzyme reactor could perform well after 260 h operation time.

Table 17 Responses of a flow-injection urea sensor system at different operation time of the enzyme reactor. (NA = did not analyze)

Concentration	Conductivity change (V)					
(mM)	1st condition	2nd condition	3rd condition	4th condition	5th condition	6th condition
10	0.05	0.06	0.06	0.05	0.06	0.05
20	0.07	0.08	0.08	0.08	0.09	NA
30	0.09	0.11	0.11	0.11	0.13	0.1
40	0.14	0.15	0.15	0.14	0.15	NA
50	0.18	0.2	0.2	0.19	NA	0.19
60	0.2	0.24	0.25	0.24	0.25	NA
70	0.23	0.28	0.29	0.28	NA	0.25
80	0.27	0.31	0.31	0.31	NA	NA
90	0.32	0.36	0.36	0.35	NA	0.35
Slope	0.0034	0.0039	0.0039	0.0039	0.0037	0.0038
r^2	0.9891	0.9936	0.9909	0.9941	0.9819	0.9889

Condition	Flow rate of the buffer line ($ml\ min^{-1}$)	Flow rate of the sample line ($ml\ min^{-1}$)	Sample volume ml	Operation time h
1^{st}	0.50	0.50	0.50	35
2^{nd}	0.50	0.25	0.50	47
3^{rd}	0.50	0.25	0.50	70
4^{th}	0.50	0.25	0.25	103
5^{th}	0.50	0.25	0.25	196
6^{th}	0.50	0.25	0.25	263

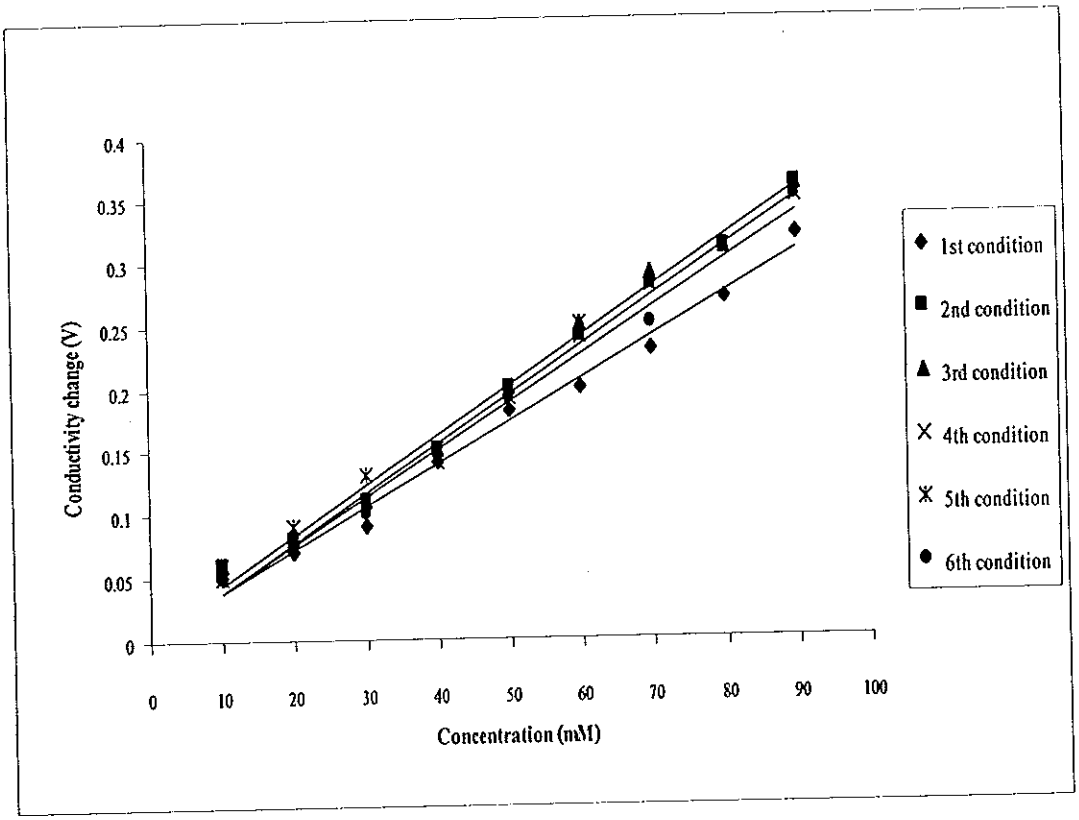

Figure 27 Response of a flow-injection urea sensor system at different operation time of enzyme reactor

Condition	Flow rate of the buffer line ($ml\,min^{-1}$)	Flow rate of the sample line ($ml\,min^{-1}$)	Sample volume ml	Operation time h
1st	0.50	0.50	0.50	35
2nd	0.50	0.25	0.50	47
3rd	0.50	0.25	0.50	70
4th	0.50	0.25	0.25	103
5th	0.50	0.25	0.25	196
6th	0.50	0.25	0.25	263

3.6 Determination of urea in serum samples

3.6.1 Berthelot reaction

The urea concentrations were evaluated by the laboratory of Songklanagarind Hospital and the results are shown in Table 18.

Table 18 Urea concentration obtained by Berthelot reaction.

Sample ID	Date dd/mm/yy	Urea concentration		Sample ID	Date dd/mm/yy	Urea concentration	
		(mM)	BUN (mg%)			(mM)	BUN (mg%)
A1	27/11/41	**7.38**	20.65	E1	20/1/42	**7.00**	19.60
A2	27/11/41	**6.33**	17.72	E2	20/1/42	**14.20**	39.76
A3	27/11/41	**12.34**	34.55	E3	20/1/42	**10.60**	29.68
A4	27/11/41	**6.51**	18.23	F1	30/1/42	**26.30**	73.64
A5	27/11/41	**10.79**	30.21	F2	30/1/42	**8.50**	23.80
B1	2/12/41	**59.50**	166.60	F3	30/1/42	**5.10**	14.28
B2	2/12/41	**61.00**	170.80	F4	30/1/42	**12.70**	35.56
B3	2/12/41	**44.70**	125.16	G1	5/2/42	**14.20**	39.76
B4	2/12/41	**67.20**	188.16	G2	5/2/42	**7.40**	20.72
B5	2/12/41	**43.50**	121.80	G3	5/2/42	**9.60**	26.88
C1	15/12/41	**51.00**	142.80	G4	5/2/42	**5.70**	15.96
C2	15/12/41	**30.80**	86.24	G5	5/2/42	**10.30**	28.84
C3	15/12/41	**76.20**	213.36	H1	20/2/42	**9.10**	25.48
D1	7/1/42	**9.90**	27.72	H2	20/2/42	**8.50**	23.80
D2	7/1/42	**10.20**	28.56	H3	20/2/42	**12.70**	35.56
D3	7/1/42	**7.40**	20.72	H4	20/2/42	**14.20**	39.76
D4	7/1/42	**11.20**	31.36	H5	20/2/42	**7.40**	20.72
D5	7/1/42	**7.20**	20.16	H6	20/2/42	**9.60**	26.88

3.6.2 Conductometric urea biosensor

The measurement of blood urea was carried out under the optimum conditions; large diffusion area dialyser, flow rate of buffer and sample lines were 0.25 ml min^{-1}, and sample volume was 0.25 ml. The results of the calibration solutions were shown in Table 19 and Figure 28. Urea concentrations in serum samples obtained from the calibration were shown in Table 20.

Table 19 The responses to urea calibration solutions of the coductometric urea biosensor system.

Urea concentration (mM)	Conductivity change (V)				
	First	Second	Third	mean	SD
0.5	0.017	0.018	0.025	0.020	0.004
1	0.053	0.048	0.048	0.050	0.003
2	0.135	0.125	0.138	0.133	0.007
3	0.248	0.241	0.250	0.246	0.005
4	0.351	0.342	0.360	0.351	0.009
5	0.416	0.425	0.420	0.420	0.005
6	0.538	0.520	0.532	0.530	0.009
7	0.630	0.620	0.618	0.623	0.006
Slope				0.0944	0.008
r^2				0.9977	

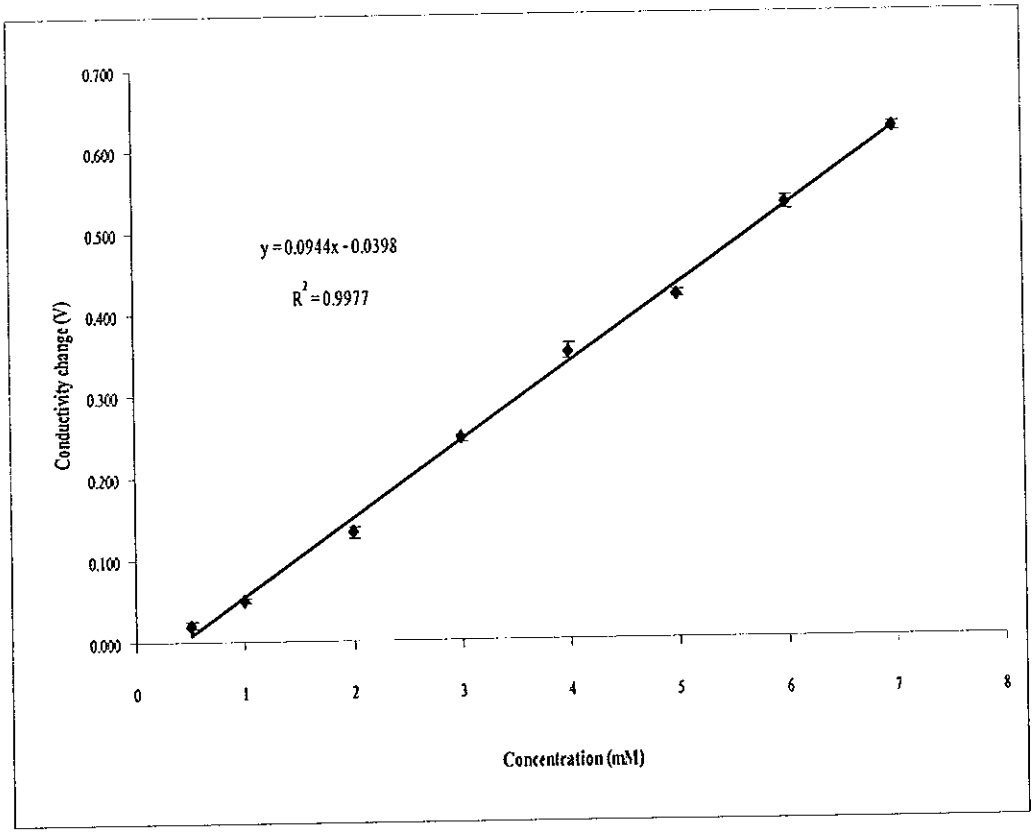

Figure 28 Calibration curve of the conductometric urea biosensor system.

3.6.3 Blood urea nitrogen (BUN) test kit (Sigma, procedure 535)

The results of the calibration were shown in Table 21 and Figure 29. Urea concentrations in serum samples obtained from the calibration equation were shown in Table 22.

Table 21 Absorption of urea standard solutions obtained with Fearon reaction.

Concentration (mM)	% T				Absorbance
	1st	2nd	3rd	mean	
0	100	100	100	100.00	0.00
5	50.2	49.8	50.6	50.20	0.30
10	25.3	25.5	25	25.27	0.60
15	13	13.4	13.6	13.33	0.88
20	6.2	5.7	6.1	6.00	1.22
molar absorbtivity					60.4
r^2					0.9988

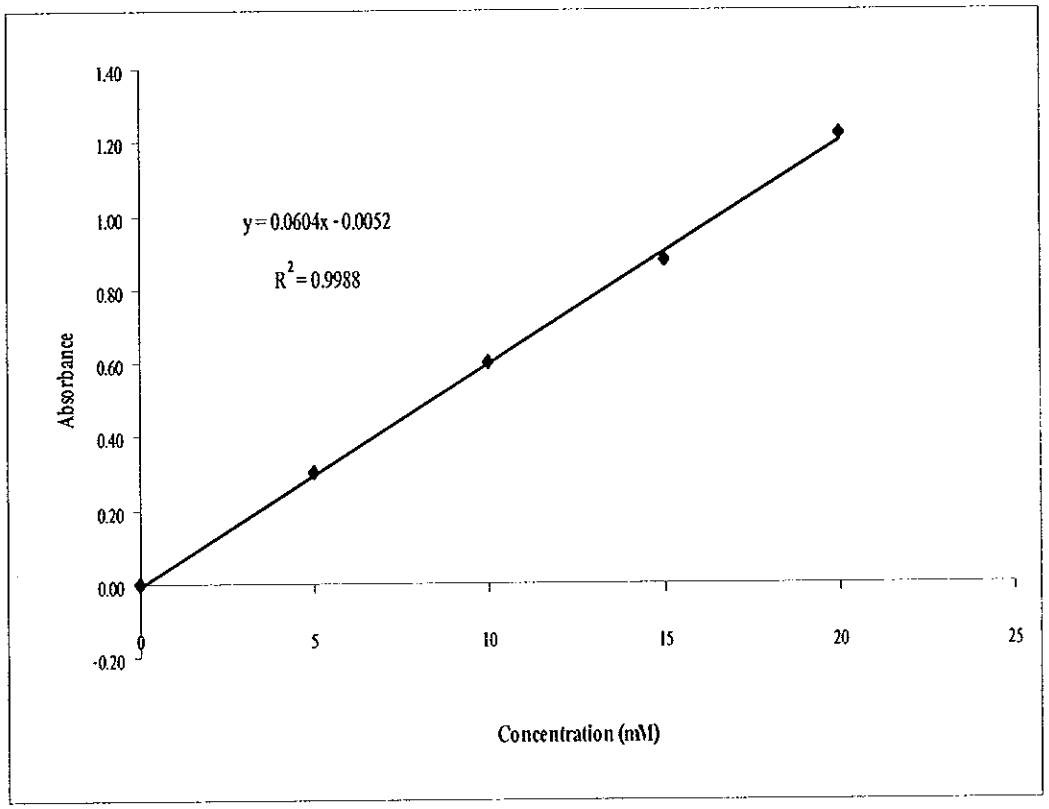

Figure 29 Calibration of urea standard solutions obtained with the Fearon reaction.

Table 22 Urea concentrations in serum samples obtained with the Fearon reaction.

Sample ID	Date	Urea concentration (mM)	Sample ID	Date	Urea concentration (mM)
D1	7/1/42	**10.1**	G1	5/2/42	**14.6**
D2	7/1/42	**10.6**	G2	5/2/42	**7.2**
D3	7/1/42	**7.4**	G3	5/2/42	**9.6**
D4	7/1/42	**11.5**	G4	5/2/42	**5.5**
D5	7/1/42	**7.0**	G5	5/2/42	**10.7**
E1	20/1/42	**7.0**	H1	20/2/42	**8.8**
E2	20/1/42	**14.6**	H2	20/2/42	**8.2**
E3	20/1/42	**11.0**	H3	20/2/42	**13.0**
F1	30/1/42	**27.0**	H4	20/2/42	**14.1**
F2	30/1/42	**8.8**	H5	20/2/42	**7.2**
F3	30/1/42	**5.4**	H6	20/2/42	**9.3**
F4	30/1/42	**12.4**			

3.6.4 Comparison of the results using Conductometric biosensor, Berthelot reaction and blood urea nitrogen (BUN) test kit

Discrete analysis validation was done with 36 samples. The results obtained with the conductometric biosensor (y) were compared with values obtained with the Berthelot reaction (x) in Figure 30. Least-squares statistical results show that the correlation between the two methods can be expressed as; $y = (0.9994 \pm 0.0023) x + (-0.1041 \pm 0.25)$, $r^2 = 0.9993$. The calculated slope (m) and intercept (C) do not differ significantly from the ideal value of 1 and 0 respectively, thus there is no evidence for systematic differences between the two methods.

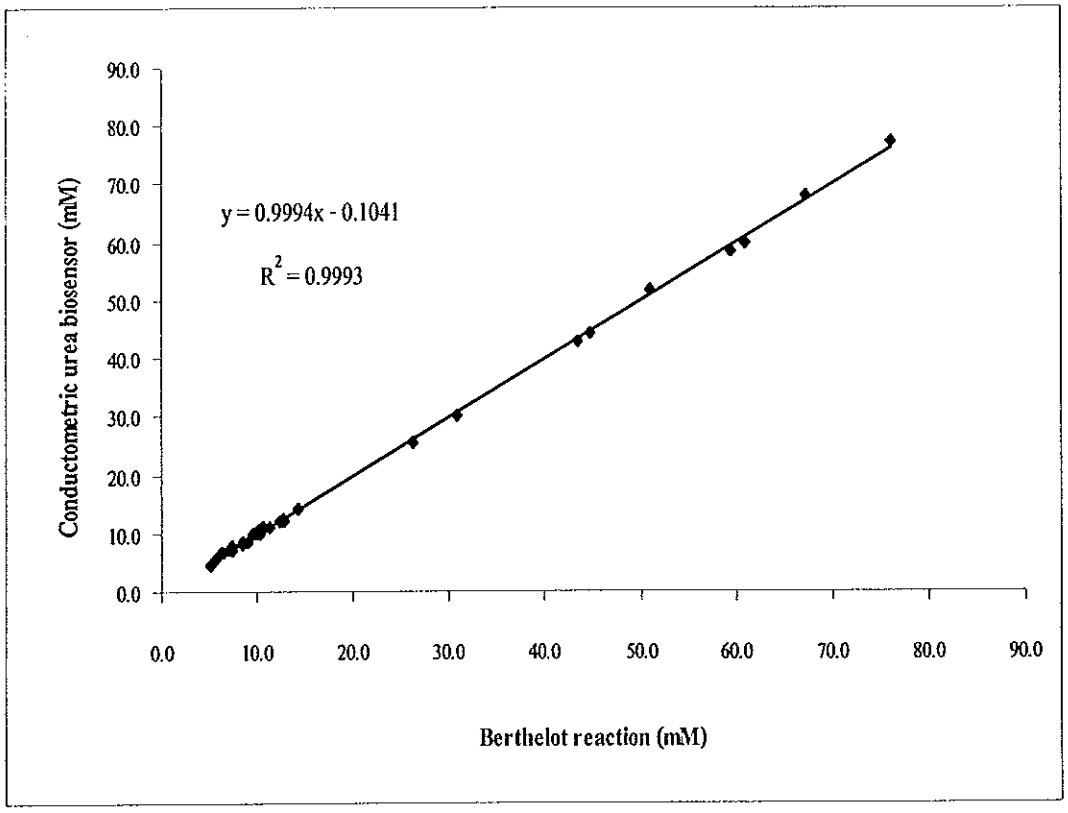

Figure 30 Correlation of the concentration of urea in serum samples obtained from the conductometic urea biosensor and the Berthelot reaction.

Table 23 shows the application of the Wilcoxon signed rank test. The sum of the positive and negative ranks are 420.5 and -186.5 respectively. Therefore, the test statistic is 186.5. From Table 1, for n = 36 the test statistic has to be $\leq$ 180 before the null hypothesis can be rejected at the significance level P = 0.05. In the present case, the null hypothesis must be retained, hence no evidence for a systematic difference between the condutometric biosensor and the Berthelot reaction methods.

Table 23 Application of the Wilcoxon signed rank test for the comparison of the concentration of urea in serum samples from the conductometric urea biosensor and the Berthelot reaction.

Sample ID	Urea concentration (mM) obtained with		The difference	Rank
	Berthelot reaction	Conductometric urea biosensor	of two method (mM)	
A1	7.4	7.8	-0.4	-19.5
A2	6.3	6.8	-0.4	-19.5
A3	12.3	12.0	0.4	19.5
A4	6.5	6.8	-0.3	-13.5
A5	10.8	10.9	-0.1	-4
B1	59.5	58.3	1.2	36
B2	61.0	60.0	1.0	35
B3	44.7	44.2	0.5	25.5
B4	67.2	68.0	-0.8	-31.5
B5	43.5	42.8	0.7	30
C1	51.0	51.6	-0.6	-28.5
C2	30.8	30.0	0.8	31.5
C3	76.2	77.2	-1.0	-35
D1	9.9	10	-0.1	-4
D2	10.2	10.5	-0.3	-13.5
D3	7.4	7.5	-0.1	-4
D4	11.2	11	0.2	8.5
D5	7.2	7.5	-0.3	-13.5
E1	7.0	7.0	0.0	-1.5
E2	14.2	14.0	0.2	8.5
E3	10.6	11.0	-0.4	-19.5
F1	26.3	25.4	0.9	33
F2	8.5	8.5	0.0	1.5
F3	5.1	4.6	0.5	25.5
F4	12.7	12.2	0.5	25.5
G1	14.2	14.0	0.2	8.5
G2	7.4	7.0	0.4	19.5
G3	9.6	10.0	-0.4	-19.5
G4	5.7	5.5	0.2	8.5
G5	10.3	10.0	0.3	13.5
H1	9.1	8.5	0.6	28.5
H2	8.5	8.0	0.5	25.5
H3	12.7	12.5	0.2	8.5
H4	14.2	14.0	0.2	8.5
H5	7.4	7.0	0.4	19.5
H6	9.6	10.0	-0.4	-19.5
Sum of posititive ranks				420.5
Sum of nagative ranks				-186.5

To compare the results obtained from the proposed method and Fearon reaction, Figure 31 and Table 24 were constructed with results from 23 serum samples. The correlation equation was $y = (0.9413 \pm 0.0720)\ x + (0.4914 \pm 1.24)$, $r^2 = 0.9891$, showing good agreement between the methods. For the Wilcoxon signed rank test, the test statistic is 80.5. For $n = 23$ the test statistic has to be ≤ 70 before the null hypothesis can be reject at significance level $P = 0.05$. It can be concluded that there is no significant difference between the result of the conductometric biosensor and BUN test kit method (Sigma, procedure 535).

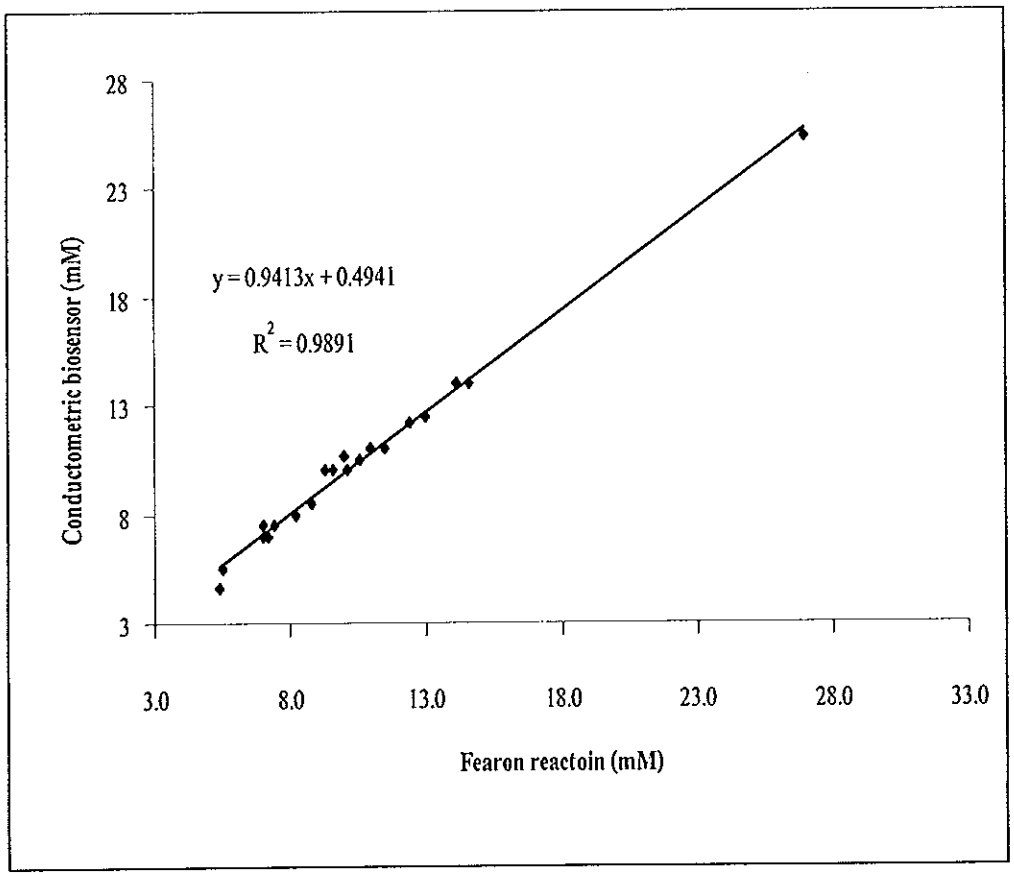

Figure 31 Comparison the concentration of urea in serum samples obtained from the conductometric urea biosensor and the Fearon reaction.

Table 24 Application of the Wilcoxon sign rank test for the comparison of the concentration of urea in serum samples from the conductometric urea biosensor and the Fearon reaction.

Sample ID	Urea concentration (mM) obtained with		The difference of two method (mM)	Rank
	Fearon reaction	Conductometric urea biosensor		
D1	10.1	10	0.1	5.5
D2	10.6	10.5	0.1	5.5
D3	7.4	7.5	-0.1	-5.5
D4	11.5	11	0.5	16
D5	7.0	7.5	-0.5	-16
E1	7.0	7.0	0.0	-2
E2	14.6	14.0	0.6	18.5
E3	11.0	11.0	0.0	2
F1	27.0	25.4	1.6	23
F2	8.8	8.5	0.3	12.5
F3	5.4	4.6	0.8	22
F4	12.4	12.2	0.2	9.5
G1	14.6	14.0	0.6	18.5
G2	7.2	7.0	0.2	9.5
G3	9.6	10.0	-0.4	-14
G4	5.5	5.5	0.0	-2
G5	10.0	10.7	-0.7	-20.5
H1	8.8	8.5	0.3	12.5
H2	8.2	8.0	0.2	9.5
H3	13.0	12.5	0.5	16
H4	14.1	14.0	0.1	5.5
H5	7.2	7.0	0.2	9.5
H6	9.3	10.0	-0.7	-20.5
Sum of posititive ranks				303.5
Sum of nagative ranks				-80.5

Chapter 4

Conclusion

A conductometric biosensor has been developed for the analysis of urea using immobilized urease on alkylamine porous glass beads. Two flow systems (flow-through and flow-injection) and two dialysers of different diffusion areas (1.5 x 49 and 1.5 x 298 mm^2) have been investigated. The optimum conditions of the flow-through system were found that to be;

- flow rate of the buffer line 0.5 ml min^{-1}
- flow rate of the sample line 0.25 ml min^{-1}
- sample volume 0.25 ml
- limit of detection 5 mM
- linear range 5-90 mM ($r^2 = 0.9947$)
- sensitivity 0.0033 V/mM.

For the flow-injection system the optimum flow rates of the buffer and sample lines were both 0.25 ml min^{-1} and the sample volume was 0.25 ml. Using these conditions the sensitivity of the responses was increased by 25% and the detection limit was lowered by 5 times from 5.0 mM to 1.0 mM. Thus, the flow-injection system was chosen for further analysis.

The large (1.5 x 298 mm^2) and the small (1.5 x 49 mm^2) dialysers were tested using the flow-injection system under optimum conditions as described above. The large dialyser gave a 20 times higher sensitivity (0.1015 *c.f.* 0.0051 V/mM), approximately 14 times higher peak height and 2 times lower limit of detection (0.5 *c.f.* 1.0 mM) than the small one. However, the small dialyser system offered a wider linear range (1 to 90 mM *c.f.* 0.5 to 10 mM) and shorter analysis time (around 15 min *c.f.* 25 min depending on the concentration). By considering the linear range and the analysis

time, the small diffusion area of dialyser may be suitable for studying factors that may affect the system. On the other hand, to measure urea in serum, the diluted samples may contained only 0.5-0.6 mM of urea concentration. This is lower than the limit of detection of the small dialyser system, but should be able to detected by the large dialyser system. So, the system with the large dialyser was chosen for blood analysis.

The effect of buffer concentrations and the stability of urea standard solutions and the enzyme reactor have also been investigated. It was found that the buffer concentration which provided enough buffering capacity for serum and offered the best sensitivity and lowest limited of detection was 0.05 M. As for the stability of the urea standard solutions prepared using glycine-NaOH buffer pH 8.8 + 0.02% sodium azide, they were found to give similar responses during the 7 days period for both storage conditions (at room temperature around 23° to 30°C and 4°C). Concerning the responses of the enzyme reactor, the wide linear range (up to 90 mM) and good sensitivity (0.0038 V/mM) can still be obtained after 263 h operation time (used intermittently over 6 months).

Taking all the above factors into consideration it was concluded that, the optimum system to determine urea in serum had to be a flow-injection system with the large dialyser where the buffer concentration is 0.05 M, the flow rates of the buffer and sample lines are both 0.25 ml min^{-1} and the injected sample volume is 0.25 ml (dilution factor was 10, so the volume of serum was 0.025 ml).

In summary, the performances of the above system were as followed;

- limit of detection 0.5 mM
- sensitivity 0.1015 V/mM
- linear range 0.5 to 10 mM ($r^2 = 0.9955$)
- stability of urea standard solution at least 7 days
- stability of enzyme reactor at least 260 h.

Analysis on the same serum samples were carried out on the autoanalyzer (Hitachi, model 717) by Songklanagarind Hospital (Berthelot reaction) and Fearon reaction. The analytical results indicate good agreement between the three methods. 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 spectrophotometic method or does it require any other pretreatment.

By comparing the proposed method to some recent works on urea biosensor techniques, it was found that the limit of detection (0.5 mM) of this system is higher than some other biosensors for urea. Detection limits of some urea biosensor systems are, amperometric ammonium ion 0.01 mM (Bertocchi and Comphgnoe, 1996), pulse-amperometric detection 0.002 mM (Adeloju *et al.,* 1997) and potentiometric ammonia electrode 0.01 mM (Liu *et al.,* 1997). However, these methods have poor selectivity, and potassium, sodium, anions and proteins may interfere with the signal. Thus, the step to remove the interferences, such as the use of anion-exchange separation, is needed (Adeloju *et al.,* 1997) while, the proposed method does not need any pretreatment.

In conclusion, the conductometric urea biosensor is a reliable sensor which can be used to determine urea in serum with accuracy. The preparation of the enzyme reactor is relatively simple and its good response stability together with the simple buffer reagent makes it more economical than the automated and spectrophotometric systems.

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Vitae

Name Mr. Woraphot Saelim

Date of Birth May 7th 1973

Academic Background

1992: High school Rattaphumvittaya School, Songkla

1996: BSc (Chemistry) Prince of Songkla University