# Investigations into the design and development of novel screen-printed electrochemical biosensors for sugars

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## Abstract

The aim of the studies described in this thesis was to develop a set of single shot biosensors, for low concentration measures (less the 1 mM) of glucose, sucrose and fructose. In addition, the development of a user friendly potentiostat instrumental system to display and save the data acquired, via the use of an android tablet.

Chapter one describes an introduction and background to screen-printed biosensors. Including the electrochemical techniques used and a basic explanation of screen printing. A brief discussion of the sugars, that will be measured in this thesis.

Chapter two describes the development of a simple, low cost chronoamperometric assay, for the measurement of fructose, using a graphite-nanoparticle modified screen-printed electrode (SPCE-G-COOH). Cyclic voltammetry showed that the response of the SPCE-G-COOH enhanced the sensitivity and precision, towards the enzymatically generated ferrocyanide species, over a plain SPCE; therefore, the former was employed in subsequent studies.

Calibration studies were carried out using chronoamperometry and tested using a commercial fruit juice.

Chapter three continues from chapter two, using studies to explore the conversion of the fructose bioassay into a fructose biosensor. More analysis of the interference of ascorbic acid (vitamin C) and a method of removing the interference signals via linear subtraction, using a secondary base electrode.

Chapter four explains measuring glucose, using a unique mediator, and measuring the amperometric signal, in the reduction phase. The chapter describes the use of multiple enzymes and the optimisation of them. This section then demonstrates the modification of the glucose biosensor to a sucrose biosensor. This was achieved by the inclusion of an invertase enzyme. The studies of the glucose and sucrose biosensor were then compared against a commercially available system.

Chapter five investigates a possible potentiostat system, linked to an android based tablet. The intention was to produce a simple and user-friendly system to measure and record the concentrations found.

Chapter six comprises future work to be carried out and ideas. These suggestions are to improve the system allowing it to be more user-friendly and to remove some of the human errors that could be introduced into the system.

## Acknowledgements

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On a personal note, I would finally like to thank my family for the emotional support throughout this time.

## Copyright Disclaimer

Contents

# Chapter 1

# Introduction

Contents – Chapter 1

This thesis describes the development of a system for the agri-food industry to monitor selected sugar levels of their produce. The system consists of three biosensors, for the sugars: glucose, sucrose and fructose. The three biosensors are based on chronoamperometric screen-printed electrodes in combination with enzymes and a mediator. For use in industry, a customised modified potentiostat will be developed along with purpose built android application is developed, to be used with three biosensors. The system is designed to be portable and used by low skilled personnel; mainly by the agri-food sector.


*Figure 1.1 Image of the portable system developed, consisting of potentiostat, tablet with built software and sensors.*

## 1.1 Biosensors

The systems described in this thesis are based on enzymatic-mediator chronoampermetric biosensors. A biosensor is defined as a device that detects or measures a biological molecule, producing a signal via a transducer. Biosensors have undergone various transformations since the introduction of them. Although there have many versions, variations and advances. [1-2]. In this thesis, the device is a screen-printed base transducer, that detects the specific sugar molecule. This presence of the sugar has an interaction with a specific enzyme and generates an electrical signal, in the form of accepting or donating an electron. The screen base transducer detects the electron difference and is measured via a potentiostat.

The advantage of using a biosensor is that it is highly specific to a specific analytes or family of analytes. The output signal can vary wildly, depending on the application. The advantage of this system is the combination of high selectivity and the integration of the system into a portable device. [3]

Glucose biosensors are the most well-known type of biosensor, commonly used by diabetic patients to monitor blood-glucose levels. The biosensor has undergone many transformations from the original concept, in 1962 by Clark and Lyons. [4] The first commercial generation of the glucose biosensors was based on the amperometric measurement (measurement of electrical current) of hydrogen peroxide. The glucose molecule is converted to its product (gluconolactone), by

glucose oxidase and the native oxygen present in the sample, also producing hydrogen peroxide. The current produced by hydrogen peroxide is directly proportional to the concentration of glucose.

There are many types of biosensors ranging from electrochemical to optical. Electrochemical biosensors convert the chemical signal to an electrical signal whereas optical converts the chemical system to an electromagnetic (typical visible light or ultra-violet light) signal [5,6].

### 1.2 Screen-Printed Base Transducers for Electrochemical Biosensors

The biosensors investigated in this thesis is constructed using screen-printed base transduces. Screen-printed base transducers, for electrochemical biosensors, are a popular production method [7-9]. This is due to the fast production of screen printing and typically relatively low production costs. One of the most common versions of screen-printed base transducers are screen-printed carbon electrodes (SPCE). An SPCE is generally comprised of the working area constructed with a carbon ink. To reduce the costs further the counter and reference electrodes can be combined, forming a two-electrode system, although this may have an impact on the sensitivity of the system. To further reduce the manufacturing costs, carbon ink maybe used for the counter-reference electrode, instead of more expensive materials such as platinum or silver-silver chloride. One major advantage of using carbon for the working and counter-reference electrode is that both can be simultaneously printed using a single screen [10-11].

## 1.3 Screen Printing

Screen-printing is the process of transferring paste, through meshed screen, onto a substrate [12]. The advantage of this process is that both the design and materials used, to create the electrodes, can be easily modified. The type of materials used can have advantageous effects to the sensor produced, in terms of electrochemical behaviour. A variety of modifications can be made such as incorporating materials such as platinum, graphite and gold incorporated. Other modifications, relating to the electrode design, include changing from a two-electrode system, comprising of a working electrode and a shared counter-reference electrode, to a three-electrode system, comprising of individual working, reference and counter electrodes.

The process of screen printing involves firstly supporting the mesh screen via a frame.

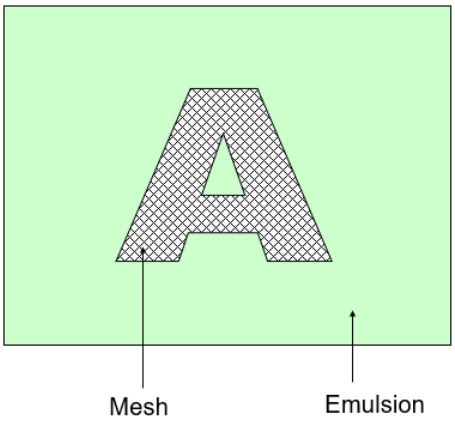


*Figure 1.1 Diagrammatical representation of screen with mesh and applied emulsion*

The mesh may be made from various materials, such as silk, polyester, stainless steel, etc. During the manufacturing of the screen, the mesh is stretch under tension and adhered to a suitable frame.

The mesh is then coated using an emulsion, as shown in Fig 1.1, the coating is carefully controlled to ±2 µm. The coating must be controlled as this contributes to the final cured thickness of the screen-printed ink.

A photopostive, a single coloured image (normally black) on a transparent substrate, is placed on top of the emulsion. The screen and photopositive are then exposed to UV-light. Any areas of the emulsion not blocked by the image of the positive becomes cured by the UV-light. Any areas blocked from the UV-light are not cured. The screen is then washed, typically with water, any non-cured areas are removed by the washing stage. This results in only the cured areas remaining, the image of the photopostive is present on the screen as mesh not coated with the emulsion.

The emulsion results in parts of the mesh are blocked. During the screen-printing process, the paste only passes through the exposed mesh (and not the mesh coated with emulsion). The paste that passes through the mesh is transferred

directly onto a substrate, resulting in the design of the mesh being present on the substrate.

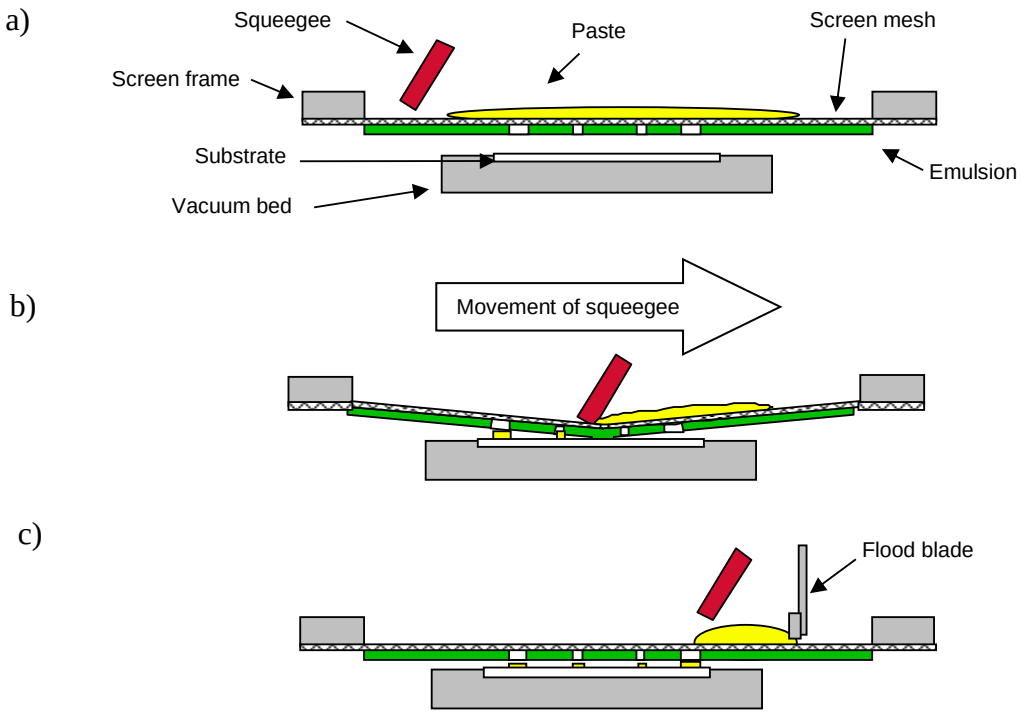



*Figure 1.2 schematic representation of the screen-printing procedure*

Fig 1.2 shows a schematic representation of screen printing: a) The figure shows the initial set up of the screen printing, with the paste spread over the surface of the screen. The spreading over the surface is caused by the flood blade (not pictured). The substrate is supported under the screen, with the aid of a vacuum bed. B) The squeegee passes over the screen, forcing the paste through the screen, (and also) making a physical connection with the substrate. Thus, the paste is transferred onto the substrate. This results in the paste remaining on the

substrate in the desired pattern of the screen. C) The paste is then spread back over the surface of the screen via the flood blade, ready for the next substrate.

Screen-printed electrodes have become more common in recent times, this is down to the low cost of the electrodes and the speed to produce the electrodes. Other methods such as spray, require longer to produce.

There are limits to the definition provided to screen printing, around 100 μm gaps and line (the printing width of gaps between printed areas and the minimum width that can be achieved). This is also dependent on the screen mesh and the type of ink used. For this thesis, the printing areas are relatively larger (in comparison to 100 μm) and therefore screen-printing is a suitable approach.

## 1.4 Screen-Printing Inks

Screen printing inks are specifically designed with the intention of being screen-printed, in the “wet” state, and to provide a functionalised structure on a substrate, when in the “cured” state.

A typical ink is comprised of three components: the functional system, the carrier system and the solvent system [12].

### 1.4.1 Function System and Materials

The functional system provides the functionality of the structure, once the ink has been cured. In the case of the working electrode material, an electrochemically active component is desired. The material could be carbon, graphite, gold, platinum, etc. Whereas the material for the insulation layer could be produced from a non-conductive material such as titanium oxide. For reference electrodes platinum or a mixture of silver and silver-chloride maybe used [13].

### 1.4.2 Carrier System and Materials

The carrier system has two functionalities. The first is to provide structure to the ink, allowing it to be screen-printed and the second is to adhere (itself and the functional system) to the substrate to which the ink is being printed onto. Examples of materials used in the carrier system are resins, such as polymers, acrylics, frit (glass), cross-linking structures, etc.

### 1.4.3 The Solvent System and Materials

The solvent system is to allow the ink to be printed, giving the ink the ability to “flow” as a liquid. An ideal solvent system provides the ink with good screen lifetime (the amount of time that the ink can be left on the screen with low evaporation rate, without compromising the flow of the ink). In additional, the solvent is required to

provide non-disruptive print film, when evaporated away. The solvent(s) used may range from polar solvents such as water to over organic solvents which are non-polar.

The solvent system is only present in the “wet” state and removed leaving on the functional and carrier systems, in the “cured” stage.

For the working electrode, there a multitude of materials that can be used, from precious metals (such as gold and platinum) to cheaper materials (such as carbon). In this thesis, carbon is used, as it is a relatively cheaper material, making the overall cost of the final biosensor more appealing to the end user. If carbon was found to not provide a sufficient signal, from the electrochemical system, then an alternative material would have been acquired.

The reference-counter electrode, in this thesis, was constructed using a silver-silver chloride material. This provides an extremely stable half-cell, where the silver chloride can be converted to silver and a chloride ion (in the presence of an electron). The opposite can apply where the system requires electrons, the silver and chloride ion can accept the electron, a produce silver-chloride.

### 1.4.4 Curing of the ink

Curing the ink from the “wet” state to the “cured” state can be carried out in numerous ways. The most common way is by applying of thermal treatment,

subjecting the print to a heat element causing the solvent system to be removed without breaking down the “carrier” system, as shown in Fig1.3.

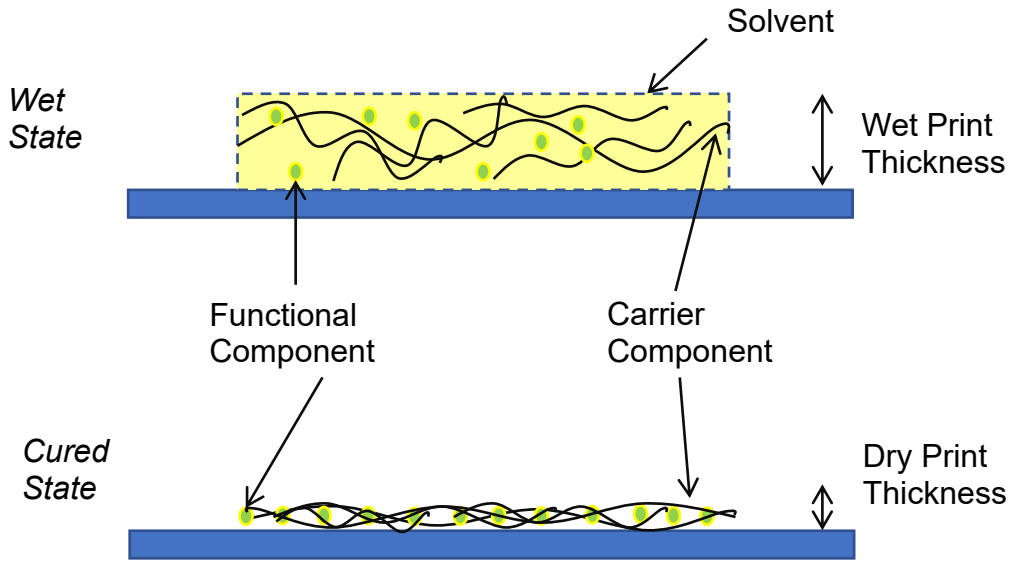



*Figure 1.3 Diagrammatic representation of a screen-printed example, in the wet state and the cured state*

## 1.5 Electrochemical Analysis Techniques

One method to measure the electrode kinetics of a target compound at an electrode is by the use of cyclic voltammetry [14-17]. This technique applies a triangular waveform as shown in Fig 1.4, which results in a peak-shaped voltagram, as shown in Figure 1.5.

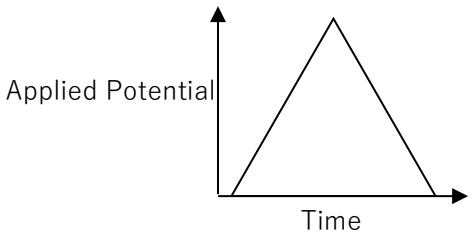



*Figure 1.4 – Graph representation of triangular waveform*

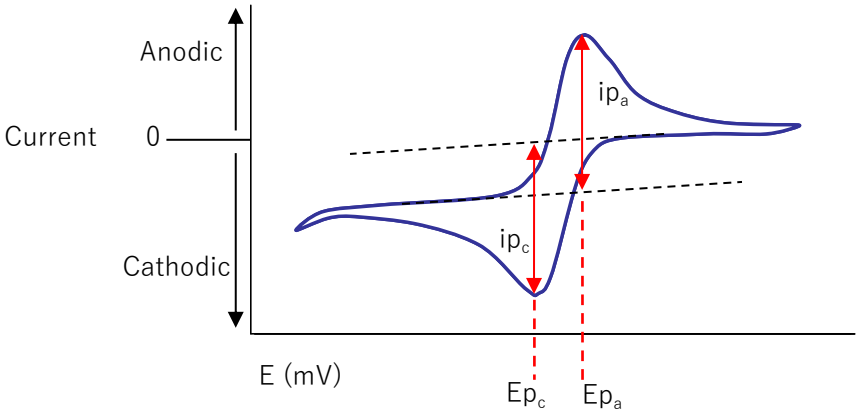



*Figure 1.5 Graph representation of standard cyclic voltammogram*

The current produced can be calculated using the Randles-Ševčík equation:

$$i_p = (2.69 \times 10^5)\, n^{3/2}\, A\, C\, D^{1/2}\, v^{1/2}$$

The equation shows how the current produced ($i_p$,A) is related to number of electrons (n), area (A,$cm^2$), concentration of the analyte (C,M) the diffusion coefficient (D,$cm^2s^{-1}$) and the scan rate (v, $Vs^{-1}$). $2.69 \times 10^5$ relates to the coefficient at 25°C.

As the potential reaches the working potential of the analyte, the current increases. The current will then start to decrease as the analyte depletes on the surface of the working electrode. At this point the current becomes limited to the diffusion of the analytes to the Nernst diffusion layer. When the potential scan is reversed, with a reversible analyte, the same happens in the opposing direction.

The graph is of a cyclic voltammogram, in non-stirred solution. When the potential is first applied, this causes the ions to be either oxidised or reduced, depending on the system. The conversion of the ions is at maximum when the optimum voltage is applied. The ions are only converted at in the Nernst layer, not the bulk solution. As this is a non-stirred solution, this layer gets replenished by the diffusion of ions, in the solution. After the current is at maximum peak height, the current starts to decline. This is due to the ions that are in the Nernst layer already being converted. The and therefore no longer give a current. The current is only produced by the ions that are surrounding the Nernst layer start to diffusion on the surface of the electrode. The more ions are converted, the more distance the non-converted ions have to diffuse to, to reach the surface of the electrode.

This is shown in the equation by the diffusion co-efficient. When the solution is stirred then the Nernst layer is replenished constantly. This produces a larger diffusion co-efficient. This results in the graph plateauing and not starting to decline, when the current peak is reached.

Most products that can be oxidised or reduced at a certain voltage. These voltages can be very high or very low, in intensity. Some species that are desired to be measured, may have a potential that is quite high. This can have problems as interference from other species with a similar oxidation or reduction potential. This will provide a signal with a lot of interference as not the desired product is being measured. A normal way of allowing the measurement of the desired species, and not the interference, is using a mediator. A mediator is a chemical species with a relatively low oxidative or reductive potential. The desired species to be measured can transfer the electrons to the mediator. Therefore, when a lower (closer to zero) potential is applied, only the electrons passed onto the mediator is measured, as a current.

There are numerous mediators that are available, the choice in mediator depends on the system. Potassium ferricyanide is a typical chemical that is used as a mediator. When a cyclic voltammogram on ferricyanide (in a buffer solution with opposing anions) is carried out, the system normally starts with a positive potential to a negative potential. As the potential is reduced the reducing potential is reach, causing the conversion of $Fe^{3+}$ to $Fe^{2+.}$ When the direction is reversed then the $Fe^{2+}$ is converted to $Fe^{3+}$.

When potassium ferricyanide is used as a mediator (in open circuit), the molecule accepts an electron, causing the species to convert from $Fe^{3+}$ to $Fe^{2+}$. When the oxidation potential is applied, then the $Fe^{2+}$ is converted to $Fe^{3+}$. The electrons

released from the $Fe^{2+}$ produces a positive current. This number of electrons is directly proportional to the number of electrodes given by the biosensor system.

Many different mediators have been found/developed. In this thesis, ferricyanide and a ferrocene-based molecule were used. The ideal mediator would have an oxidative or reductive potential close to zero, to eliminate signal produced by interference molecules. Other common mediators include Medolas Blue, where it is used in conjunction with $NADH/NAD^+$ systems. This reduces the voltage required from +0.6 V to +0.1 V (depending on materials and design of electrode), thus avoiding any possible inferences at +0.6V [18].

In the cases of a single enzyme-based biosensors, the enzyme converts the biological substrate to its products. The enzyme itself is either oxidised or reduced, depending on the system. These electrons can then be passed onto the mediator. This allows the enzyme to return to its original state, the enzyme can then be recycled back into the system, causing more conversion if the biological substrate to its product.

The biosensor can be measured in two ways: kinetically or via an end-point reaction. When a kinetical assay is carried out, the concentration of the biological substrate has not been depleted. This means that the system is very time dependent. For example, if the time was reduced, this would not convert the same amount of biological substrate to its product, therefore not generating the same

number of electrons, and so the current produced would not be the same. The same can be said if the time was increased, as this would allow the system to convert more biological substrate to its product and therefore generate mere electrons, this again would change to current that is produced.

To make the system less time dependent, an end-point reaction can be carried out. In this instance, the system is given enough time for the full conversion of the biological substrate to its product and also extra time, to remove human error, when carrying out the assay. This produces more reliable results, with less variance. The disadvantage of an end-point reaction is that the incubation time (time at open circuit) is longer, which maybe unfavourable in terms of the user.

A current is produced when the enzyme interacts with its substrate, generating an electroactive product. The current produced is proportional to the substrate concentration, see Fig 1.6

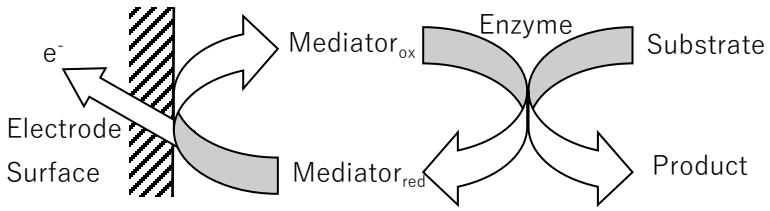



Figure 1.6 Generic scheme of electrochemical reaction

For enzyme-based biosensors, the base transduce is normally coated with the enzyme and a mediator. The enzyme and the mediator can be applied to the base

transducer in a number of manners. In some cases, the enzyme and the mediator can be integrated into the screen-printed ink, and then screen printed. This cannot be applied to may systems, due to the strains and stresses that are put onto the enzyme, normally causing the enzyme to be denatured. Also, buffer systems are required to allow the enzyme to work in optimum conditions.

In other instances, the mediator and enzyme are deposited onto the electrode, sometimes as a single solution or as separate spots/solutions. Normally the solutions are separate is the two chemicals require different optimum shelf-life conditions, or that they interact when mixed over time.

When a biosensor is first developed, a calibration curve is carried out [16]. This is achieved by having a selection of standard known concentrations is produced. This is normally done using analytical solutions. For more systems a linear graph is produced, using the different concentrations. When a linear graph is produced, the equation y=mx+c, an unknown sample can then be tested. The unknown concentration will produce a current (y). by subtracting the c from y, followed by dividing by m, the concentration (x) can be determined.

Depending on the system, the current produced can be either positive or negative. The gradient can also be positive or negative. A positive gradient normally indicates that the current produced is by the generation of a species, as a result of the desired species that is to me measured. Whilst a negative gradient normally

indicates that the species, using to produce the current, is being converted by the species that is desired to be measured.

When a negative current is produced, this is due to the cathodic current being measure, whilst a positive current is due to the measuring of the anodic current.

The position of the anodic and cathodic (on the voltage axis) is dependent on various factors. One of the factors is related to the base transducer, this may be due to the interaction between the species and the surface of the electrode. The resistance of the material, that is used to produce the base transduce.

Another characteristic that can be measured is the peak-to-peak separation. This is the distance (on the voltage axis) between the anodic and cathodic maximum peak heights.

The optimum peak-to-peak separation is -0.59 mV, this value is related to the voltage produced by a mole of electrons.

## 1.6 Reducing Sugars

### 1.6.1 Fructose

The ability to precisely and accurately measure the sugar known as fructose has become of great interest to many food companies. Industries such as those

involved in wine manufacturing need to know the concentration, of fructose (along with glucose). This is required to predict the alcohol content of the end result wine, from fermentation [19]. Sugar content can be described as one of the most desirable characteristics in commercial fruit. It can be used for processing the fruit and also provides an indication of consumption freshness [20].

Currently few reports describe the development of biosensors for the measurement of this sugar, compared with other sugars such as glucose and sucrose. One of the current methods to measure fructose and other simple sugars is by the °Brix test. This test provides the percentage of total dissolved solids. The test is based on how the light refracts through a liquid using a refractometer [21]. The Brix scale measures the total solid content of a solution at 20°C, for example, if a solution has a 10 °Bx then the solution is 10% w/w. As this method uses refraction, alcohol can vary (increase) the result due to the difference in refractive index between alcohol and water. This is a general problem when measuring foods and drinks that contain alcohol, as a result of fermentation. The fructose that is found in food products is normally from the breakdown of sucrose to its products, fructose and sucrose.

Fructose and glucose have found to be the cause of cargenogenic products when burnt.

### 1.6.2 Glucose

The issue with this biosensor is that a high potential must be applied, to detect hydrogen peroxide; this can lead to interference from other molecules. In addition, the concentration of oxygen varied between samples of biological fluids, in some cases oxygen concentration is the limiting factor. This affects the linearity of response, sensitivity and precision.

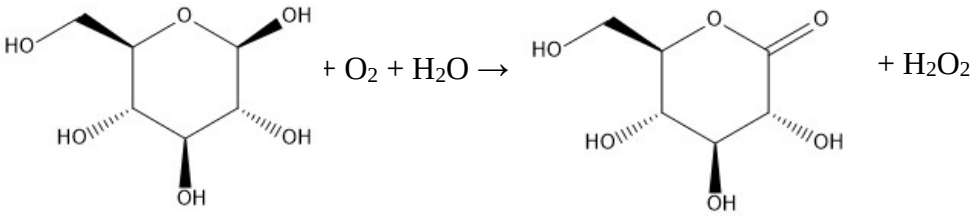
This type of biosensor operation is via the amperometric measurements [22]. This is when a voltage is applied and the current is measured against time. The current produced tends to be higher when then voltage is first supplied and decreases with time. This is due to the Nernst layer, where diffusion has an effect. The longer the time, the more the system is dependent on the diffusion co-efficient. Different biosensors read the current at different times, depending on the system. Generally, the shorter the time, that the current is read at the more sensitive the system is.

Glucose biosensors have undergone many transformations from the original concept, in 1962 by Clark and Lyons [23]. The first commercial generation of the glucose biosensor was based on the amperometric measurement of hydrogen peroxide. The glucose is converted to its product (gluconolactone), by glucose oxidase and the native oxygen present in the sample, also producing hydrogen peroxide (Fig 4.2(i)). The current produced by the oxidation of hydrogen peroxide is directly proportional to the concentration of glucose. The issue with this biosensor is that a high potential must be applied, to detect hydrogen peroxide; this

can lead to interference from other molecules. In addition, the concentration of oxygen may vary between samples of biological fluids, in some cases oxygen concentration maybe the limiting factor. This affects the linearity of response, sensitivity and precision.

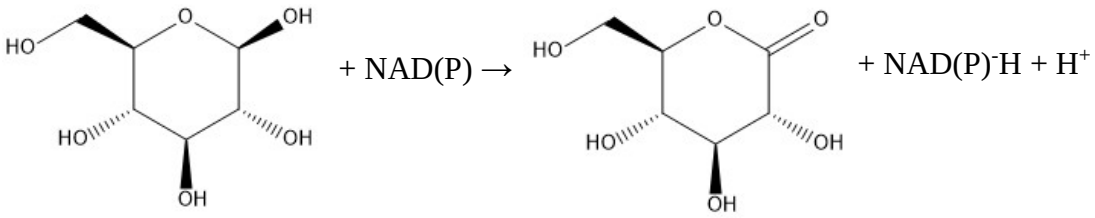
The second generation of glucose biosensors, developed in the 1980's [24], addressed the issue with the high potential required for the measurement of hydrogen peroxide, by the incorporation of a mediator, such as a ferrocene or ferricyanide. These species reduce the overpotential of the electrode reaction and allows a lower operating potential to be used. Another variation of the glucose biosensor was to change from glucose oxidase (an enzyme which was dependent on oxygen) to glucose dehydrogenase [8] (Fig4.2(ii).

i) Reaction of β-D-glucose:oxygen 1-oxidoreductase (EC 1.1.3.4)




B-D-Glucose D-Glucono-δ-lactone

ii) Reaction of β-D-glucose:NAD(P)$^+$ 1-oxidoreductase (EC 1.1.1.47)




B-D-Glucose D-Glucono-δ-lactone

*Figure 4.2 The Enzymatic Reactions of Glucose Oxidase and Glucose Dehydrogenase.*

The electron transfer process is facilitated by the mediator. This results in the current being acquired at a potential, closer to zero, for the operation of the biosensor. This reduces possible interference(s) from other naturally occurring molecules.

The third generation of glucose biosensors eliminates the use of a mediator [25], which can be. Instead the electrons are directly transferred from the enzyme to the surface of the working electrode. The disadvantage of this is that the electrode material maybe toxic and not suitable for continuous monitoring inside the body.

Another technique that has been used to measure glucose concentrations involving GOx/GDH is via UV-visible spectrometry [26]. This approach however is not practical for “in field” use. In addition, the measurements are more prone to interference. The equipment is not portable and requires calibration after being moved. A series of chemicals are required which can cause problems regarding

chemical handling, such as 4-Aminoantipyrine and direct contact with the enzyme solutions that are irritant(s).

## 1.7 Graphene/Graphite Nanoparticles

The first concept of graphene was in the 1940's, an individual planar carbon layer, of one atom thick, derived from graphite, the concept was then proved in the 1960's. In the last few years the use of graphene has come into play [27]. One aspect of the use of graphene is in electrochemical sensors. Graphene-based electrodes have been suggested to be more beneficial than carbon nanotubes due to higher macroscopic scale conductivity. It has been shown that graphene has an electrochemical potential window of 2.5V (in a solution at pH 7.5 of 0.1M phosphate buffer solution [PBS]). This makes it comparable to other materials such as glassy carbon and graphite [28]. Past experiments have shown that graphene can produce peak-to-peak separations, close to the ideal value. The ideal value of a peak-to-peak separation is 59mV, graphene can produce a 61.5-73mV for $[Fe(CN)_6]^{3-/4-}$ at 10mV/s [29].

Studies have shown that graphene can undergo direct electrochemistry of certain enzymes. One enzyme that this has been observed on is glucose oxidase. Due to graphene electrodes having a high surface area this results in high enzyme loading, therefore increasing the sensitivity [30].

The high production of graphene has been investigated. Initially, the production of graphene was acquired via a method known as the "scotch-tape" method [31]. This method was suitable for proof-of-concept but not for mass production.

It is generally believed that graphene itself is not present in sensors, as this would consist of only a monolayer of graphite. However, electrochemical (bio)sensors currently contain multiple layers of graphite and in this report the structures will be designated as graphite nanoparticles (GNPs).

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# Chapter 2

## Development of a simple, low cost chronoamperometric assay for fructose based on a commercial graphite-nanoparticle modified screen-printed carbon electrode.

Contents – Chapter 2

## 2.1 Introduction

The ability to precisely and accurately measure the sugar known as fructose has become of considerable interest, to many food companies. For example, the wine manufacturing industry use the concentration of fructose (along with glucose) to predict the alcohol content following fermentation [1,2]. The fructose concentration in commercial fruit is also an important indicator of the ripeness, of the food product [3], were has been previously done via colour determination. Colour determination can vary with variety and genetics, thus not being a suffice method. This determines when the growers can sell the product and also to make fruit juices.

Currently, few reports describe the development of amperometric assays for the measurement of fructose, compared with other sugars such as glucose and sucrose [3,4]. One of the current methods, of determining fructose and other simple sugars, involves the °Brix test [5] which is based on refractometry; this provides the percentage of total dissolved solids present in the liquid sample. As this method involves refractive index measurements, alcohol can have a detrimental effect on the result, owing to the difference in refractive index between alcohol and water [6]. An alternative approach is based on fourier transform infrared [7,8], however this technique is not readily applicable to remote analysis and has a relatively high cost.

An attractive alternative approach, that is explored in this chapter, involves the development of a simple chronoamperometric assay, based on a screen-printed

electrode. This is a low-cost method, particularly when carbon materials are used in the fabrication of the electrodes. Screen-printed carbon-based sensors have been previously developed by our group for the measurement of a wide variety of analytes, [9,10]. We recently demonstrated the possibility of measuring the sugar galactose, using the enzyme galactose oxidase in conjunction with a screen-printed carbon electrode, modified with the mediator cobalt phthalocyanine [11,12]. In another paper, we demonstrated the possibility of developing a biosensor for the measurement of glutamate, in a food sample using the enzyme glutamate dehydrogenase integrated with a screen-printed carbon electrode. It was possible to carry out the analysis of commercial OXO cubes, after a very simple dissolution and dilution step [13]. Consequently, we decided to explore the possibility of developing a simple electrochemical sensor system, for the measurement of fructose in food samples, based on screen-printed carbon electrodes (SPCEsand using the enzyme fructose dehydrogenase. As the incorporation of nanoparticles in the chronoamperometric measurement of glutamate proved to be advantageous, we decided to investigate a novel nanomaterial, nano-graphite particles, in the present study.

This paper describes the optimization of the components and operating conditions, of a chronoamperometric assay for fructose; this incorporated fructose dehydrogenase with a nanoparticle modified screen-printed electrode. The possibility of measuring the sugar, in a commercial fruit juice, will be demonstrated.

## 2.2 Experimental

### 2.2.1 Chemical reagents

D-fructose dehydrogenase was obtained from Toyobo Enzymes (Japan). (www.toyobo-global.com)

The graphite-nanoparticles (graphite modified with carboxylic acid linkages) in solution (C2131210D1) were obtained from Gwent Electronic Materials. (www.gwent.org).

Apple juice from concentrate was obtained from Tesco, UK.

All other chemicals and reagents were obtained from Sigma-Aldrich (UK). (*www.sigmaaldrich.com)*

McIlvaine buffer was prepared by mixing 0.2 M citric acid (containing 0.2 M KCl) with 0.4 M disodium phosphate (containing 0.2 M KCl) to produce a final pH of 4.5.

### 2.2.2 Apparatus and Instrumentation

All electrochemical measurements were conducted with a two-electrode system, consisting of a screen-printed working electrode (GEM code: C2030519P4), Ag/AgCl reference electrode (GEM Product Code: C61003P7) both screen-printed onto valox (a semi-crystalline material based on polybutylene terephthalate and polyethylene terephthalate polymers; Cadillac Plastics Swindon, UK). The diameter (6 mm) of the working electrode was defined using a dielectric ink (GEM Product Code: D2070423P5) a concentric silver/silver-chloride served as the counter/reference electrode, (GEM Electrode Design: BE2110916D1). For further

studies, the surface of the working electrode was modified by addition of 10µl of graphite-nanoparticles (1.787 mg $ml^{-1}$) (GEM code: C2131210D1).

The working and reference electrodes were connected to the potentiostat with GEM electrode connector (GEM Code: CON002), an edge connector with a 2.54 mm pitch. All electrochemical studies were performed using an AutoLab [µAutoLab Type II], Metrohm, with General-Purpose Electrochemical Software (The Netherlands). Data were further analyzed with Microsoft Excel.

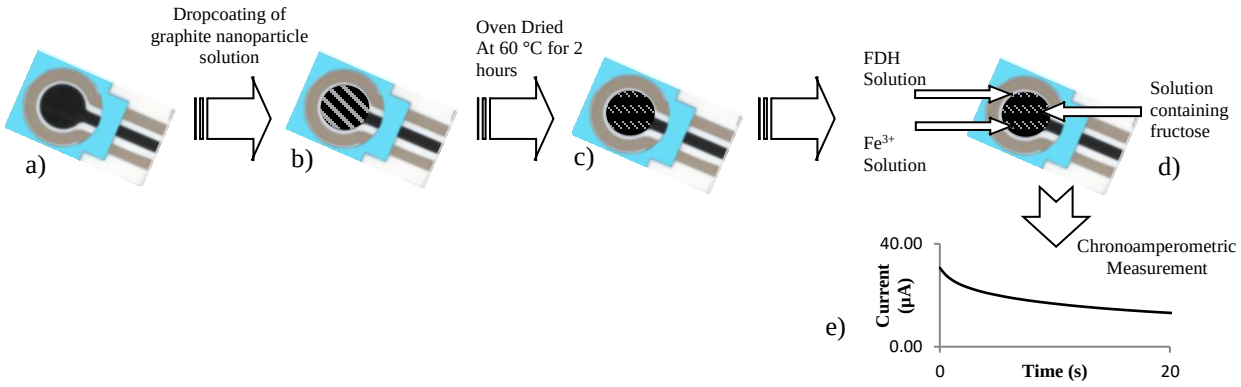


*Figure 2.1 Scheme showing the fabrication of the fructose biosensor and chronoamperometric measurement of fructose: a) Plain SPCE; b) SPCE with deposition of nanoparticles in solution; c) SPCE with dried nanoparticles; d) addition of 10 µl FDH, 10µl ferricyanide; 20 µl of solution containing fructose; e) Chronoamperometric measurement of +0.3 V, with reading at 20 s after a 3 minute incubation period of open circuit.*

### 2.2.3 Procedures

Cyclic voltammetry was performed by depositing a 300 µl aliquot of 0.5mM ferriycanide, in 0.1 M phosphate buffer pH 7.5 containing 0.1 M potassium chloride onto the surface of the screen-printed carbon electrodes. Cyclic voltammetry was performed using the following conditions: initial and final potential +0.8 V; switching potential –0.4 V; scan rate 10 mV $s^{-1}$. Potential held at +0.8 V for 20 seconds before initial cycle.

Calibration studies were performed using chronoamperometry with standard solutions of fructose, over the concentration range 0.20 mM to 32.00 mM, in water; FDH was dissolved in McIlvaine buffer to produce concentrations of either 50 U $ml^{-1}$ or 200 U $ml^{-1}$. The measurement procedure involved the deposition of 20 µl of either enzyme solution, onto the screen-printed transducer, followed by 10 µl of 12 mM ferricyanide and 10 µl fructose standard. Following an incubation time of 180 s (open circuit), with initial 20 s of agitation, the potential was stepped from open circuit to +0.3 V vs Ag/AgCl. Currents were measured 20 s after application of the voltage and these values were used to plot calibration graphs.

### 2.2.4 Analytical Application

A preliminary study was performed with the commercial apple juice, to deduce an appropriate dilution procedure. A series of dilute apple juice solutions were prepared by mixing the neat sample with deionized water to produce final dilutions in the range of 1/2 and 1/512, of the original juice, of unknown concentration. The

analysis was carried out using chronoamperometry as described above, and from the results the optimum dilution that produced a signal within the linear range was deduced.

The method of standard addition was performed with the optimum dilution of the apple juice (with deionized water). This was achieved by mixing the diluted apple juice with different concentration fructose standards, so that the final concentration of the standard added was between 0.1 and 0.8 mM, with each data point being repeated 5 times. The concentration of fructose in the original sample was obtained from this data, together with the precision of the measurements.

The recovery of added fructose was ascertained by spiking the original sample with 477.2 mM of fructose (this was equal to the concentration found in the undiluted sample). The same chronoamperometric procedure was used, as previously described.

## 2.3 Results and Discussion

### 2.3.1 Principles of the Amperometric Measurement of Fructose

$$\text{Fructose} + FDH_{Ox} \rightarrow FDH_{Red} + \text{5-dehydro-D-fructose} \quad \text{(Equation 1)}$$

$$FDH_{Red} + 2Fe^{3+} \rightarrow 2Fe^{2+} + FDH_{Ox} \quad \text{(Equation 2)}$$

$$2Fe^{2+} \rightarrow 2Fe^{3+} + 2e^{-} \quad \text{(Equation 3)}$$

$FDH_{Ox}$ = Oxidised Fructose dehydrogenase

$FDH_{Red}$ = Reduced Fructose dehydrogenase

$Fe^{3+}$ = Oxidised Iron Molecule (in the form on ferricyanide)

$Fe^{2+}$ = Reduced Iron Molecule (in the form on ferrocyanide)

Equations (1)-(3) summarise the sequence of reactions involved in the chronoamperometric measurement of fructose, at the surface of a SPCE-G-COOH. Initially, D-fructose dehydrogenase ($FDH_{Ox}$) is reduced by fructose (eqn1) and this reduced form of the enzyme ($FDH_{Red}$) reduces ferricyanide ($Fe^{3+}$) to ferrocyanide ($Fe^{2+}$) (eqn2); the oxidized form of the enzyme ($FDH_{Ox}$) is regenerated during the latter reaction. At a potential of +300 mV, ferrocyanide is oxidised back to ferricyanide (eqn3), resulting in the analytical response. Prior to the application of the applied potential, an incubation time of 3 minutes is allowed for the enzymatic oxidation of fructose; the reaction involving ferricyanide results in the conversion of two molecules of the mediator for every molecule of fructose. The magnitude of the resulting electrocatalytic current is proportional to the concentration of fructose, over the range of interest.

### 2.3.2 Cyclic Voltammetric Behaviour

An initial study was performed in order to investigate the effect of modifying the SPCE surface with COOH-graphite nanoparticles. The nanoparticles were drop coated onto the plain screen-printed electrode (and dried) and interrogated by cyclic voltammetry, using a solution of 0.5mM ferricyanide, in 0.1 M phosphate buffer pH 7.5 containing 0.1 M potassium chloride.

Fig.3.2 shows a comparison of the cyclic voltammetric behaviour of an unmodified screen-printed carbon electrode (a) and a SPCE with 17.89 µg deposited onto the surface (b). The increase in current magnitude may be explained by an enhancement in the electron transfer properties, from ferrocyanide to the modified electrode surface. It should be mentioned that the coefficient of variation was determined for the anodic peak currents; this was found to be reduced from 6.73% to 4.75% (n=9), for plain SPCE and SPCE-G-COOH, respectively. This For the development of the chronoamperometric assay this improved precision, together with the improved sensitivity, can be considered of importance to the development of a reliable analytical procedure.

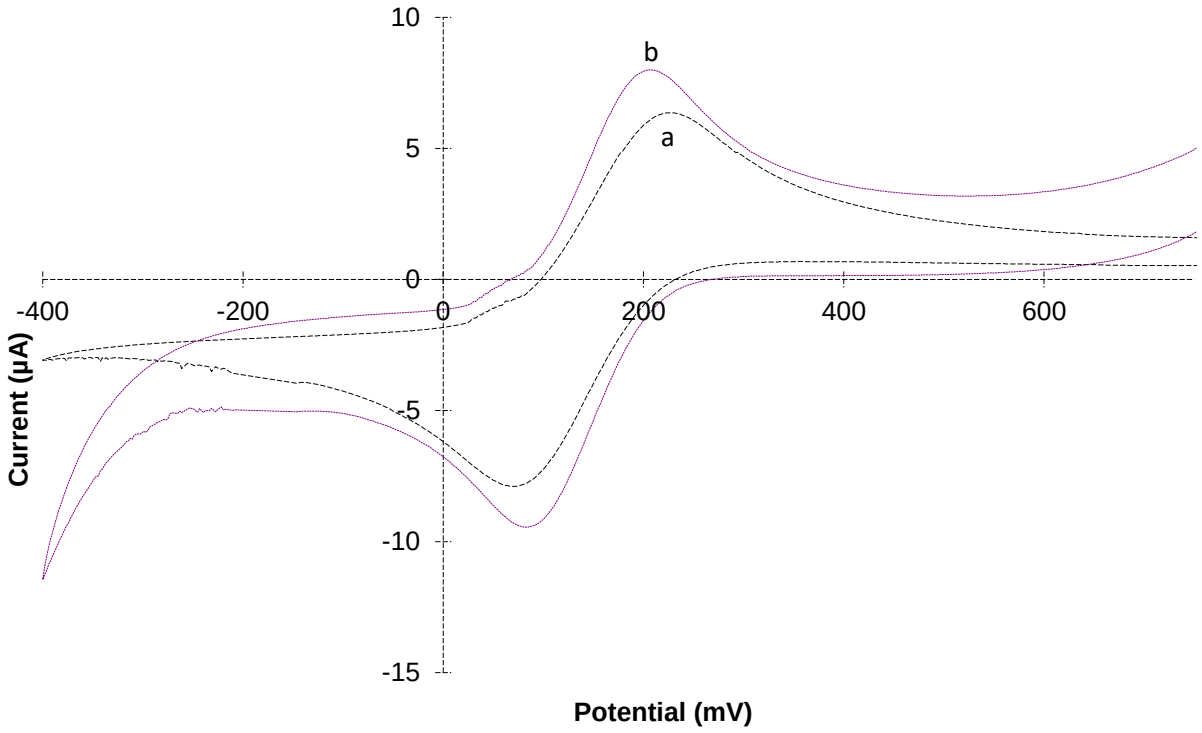


*Figure 2.2 A typical cyclic voltammograms obtained with 0.5 mM ferricyanide in 0.1 M phosphate buffer pH 7.5 containing 0.1 M potassium chloride using a scan rate of 10 mV $s^{-1}$: for (a) plain SPCE (b) SPCE-G-COOH. Voltammetric conditions: starting potential +0.8 V, switching potential -0.4 V.*

Fig.2.2 also shows that there is a difference in the peak separation ($\Delta E_p$) for the two voltammetric scans, for the plain SPCE and SPCE-G-COOH; these values were 169 mV and 148 mV, respectively. Both values indicate that the redox reaction is quasi-reversible; however, the reaction at the SPCE-G-COOH appears to be more favourable. Further evidence for was obtained the charged transfer coefficient ($\alpha$), using equation 4 [14] where *n* is the number of electrons involved in the rate determining step. The values were compared to the theoretical electron transfer voltage of 0.048V.

$$\alpha n = \frac{0.048}{E_p(V) - E_{\frac{p}{2}}(V)}$$

(Equation 4)

$\alpha$ = charged transfer co-efficient

$n$ = number of electrons involved in rate determination

$E_p$ = difference between reduction and oxidation potential

The $\alpha$ values (obtained using n=1) for the plain SPCE and SPCE-G-COOH were calculated to be 0.56 and 0.64, respectively. Therefore, the electron transfer kinetics are more favourable in the case of the SPCE-G-COOH, which indicates that this sensor has superior electrochemical characteristics, which should lead to more reproducible measurements. It should be mentioned that both values are better than reported for a commercial graphite electrode (reported α = 0.486) [15]. From these results, it appears that the reason for the increase in current magnitude occurs as a result of improved electron transfer, rather than a simple increase in electrode surface. Consequently, all further studies were performed using the SPCE-G-COOH electrodes.

2.3.3 Calibration Studies, using chronoamperometry with a SPCE-G-COOH

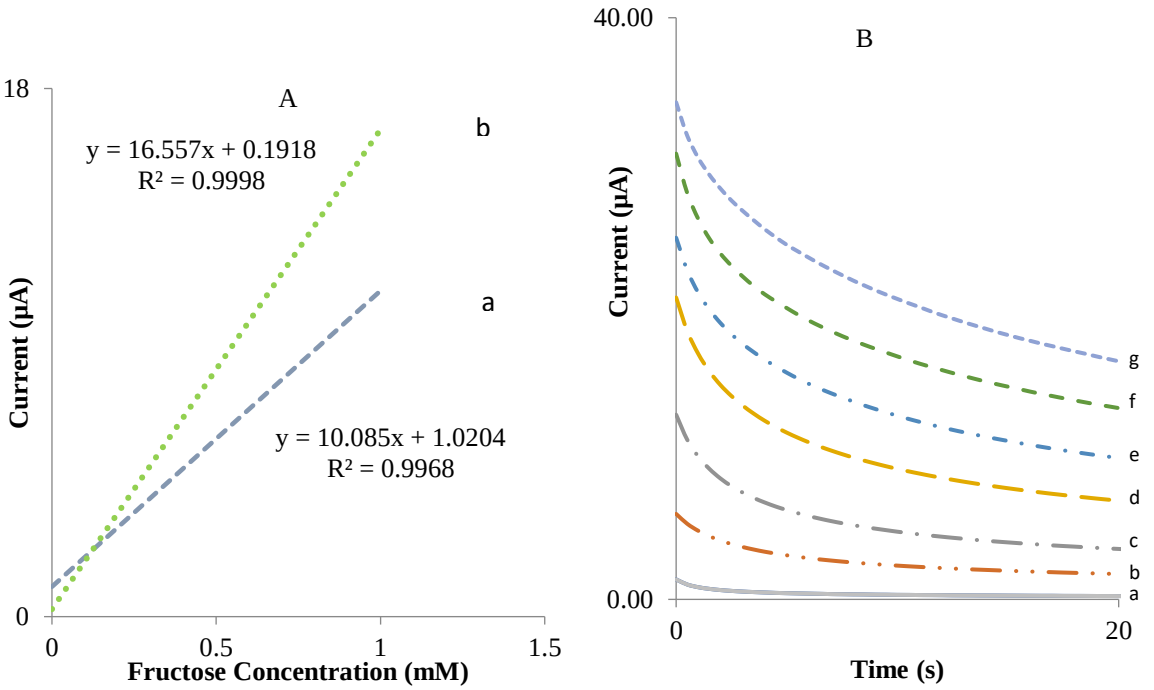


*Figure 2.2.A calibration plots obtained for fructose using a) 1 unit of FDH with a 3 minute incubation time; b) 4 units of FDH with a 3 minute incubation time.*

*Figure 2.2.B chronoamperograms obtained with the fructose sensor for various concentrations of fructose: a) 0.00 mM; b)0.10 mM; c)0.20 mM; d)0.40 mM; e)0.60 mM; f)0.80 mM; g)1.00 mM. Sensor operation performed with 4 units of FDH with a 3 minute incubation time.*

This study began by investigating the SPCE-G-COOH modified with 1 unit of FDH deposited onto the surface; a 3 minute incubation time was employed at room temperature, at open circuit. A calibration study was carried out over the range 0.10

to 1.00 mM fructose; a linear response was obtained under these conditions and the slope was found to be 10.085 μA $mM^{-1}$, Fig.2.2.A(a).

In order to investigate the possibility of increasing the sensitivity of the chronoamperometric assay, the enzyme loading was increased to 4 units, on the sensor surface. Figure 2.2.A(b) shows the resulting calibration plot, obtained under these conditions and demonstrates a linear range of up to 1 mM, with a slope of 16.6 μA $mM^{-1}$± 0.4 μA $mM^{-1}$. Figure 2.2.B shows typical chronoampergram graphs obtained for standard solutions of fructose, over the range 0.10 mM to 1.00 mM; currents were measured at 20 seconds after the potential was initiated. It should be mentioned that the sensitivity achieved with the COOH-G-SPCE was found to be higher than that reported in several papers. The following sensitivity values have been normalised from the original papers to give sensitivity μA $mM^{-1}$ $cm^{-2}$, which allows comparison with the current assay: Nicholas, et al. 2017 (current study) 58.56 μA $mM^{-1}$ $cm^{-2}$; Biscay, Rama, García, Reviejo, Carrazón, García, 2012 (ferrocyanide modified SPCE) 9.95 μA $mM^{-1}$ $cm^{-2}$; Trivedi, et al. 2009 (amperometric biosensor using FDH) 2.19 μA $mM^{-1}$ $cm^{-2}$; Antiochia, et al., 2014 (osmium-polymer mediated biosensor) 1.95 μA $mM^{-1}$ $cm^{-2}$. One possible reason for the large increase in sensitivity is due to the proposed bioassay being an endpoint reaction. Thus, all the reactions have occurred and therefore not dependent on the enzyme kinetic rates. This allows the maximum electron transfer to be measured, on the working electrode surface. At this point it was considered that the optimised chronoamperometric assay conditions would be suitable for the

analysis of a range of fruit juices; however, for evaluation purposes a typical commercial apple juice was selected. This is described in the following section.

### 2.3.4 Analytical Application

In order to evaluate the chronoamperometric assay for the measurement of fructose in commercial fruit juices, a typical apple juice product (obtained from a local supermarket) was analysed using the developed assay. Figure 3.3.4-1A shows typical chronoamperometric responses obtained for the samples of apple juice, diluted 500 times (with deionised water (Fig. 2.3A a) and after the addition of standard fructose solutions (Fig. 2.3A b-f). Fig.4B shows a typical standard addition plot, from which the original concentration of fructose was determined.

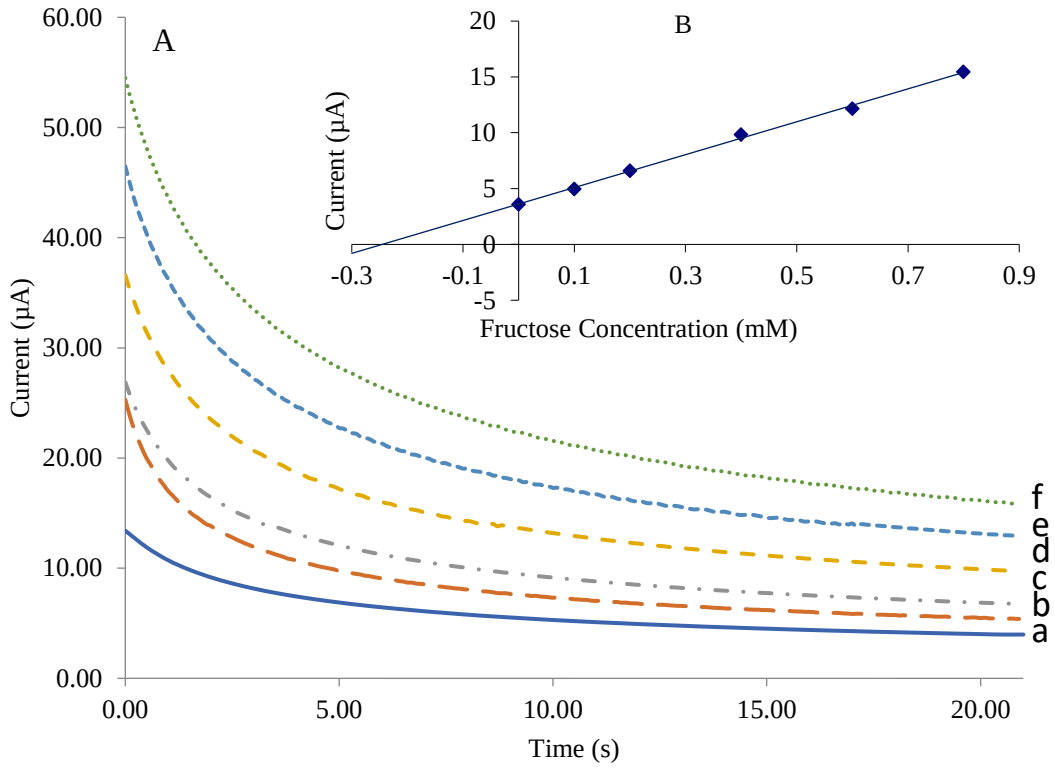


*Figure 2.3.A. Typical chronoamperogram obtained with SPCE-G-COOHs for different concentrations of D-fructose: a)Apple Juice, b)0.10 mM, c)0.20 mM, d)0.40 mM, e)0.60 mM and f)0.80 mM. Incubation time at open circuit was 180 s, followed by applied potential of + 0.3 V vs Ag/AgCl. The supporting electrolyte was 0.1 M phosphate buffer containing 0.1 M potassium chloride.*

*Figure 2.3.B. Typical standard addition plot, obtained using chronoamperometric currents measured 20 s after application of +0.3 V vs Ag/AgCl*

Table 2.1 shows a summary of the data obtained for the original fructose concentration of the unspiked apple juice. It should be noted that column 2 refers to the concentration on the screen-printed transducer; column 3 refers to the

concentration of fructose corrected for the dilution factors. As the resulting mixture on the electrode contains 10 µl of test sample and 30 µl of additional component solutions, this results in a further 4x dilution. Therefore, to calculate the original fructose concentration, the concentration determined by the bioassay requires multiplying by 2000 (from an original dilution of 500).

Table 2.1. Concentration of fructose determined in dilute fruit juice and calculated original values (n=5).

| Sample | Concentration determined after dilution (mM) | Calculated Original Fructose Concentration (mM) |
|---|---|---|
| 1 | 0.244 | 490 |
| 2 | 0.235 | 470 |
| 3 | 0.240 | 480 |
| 4 | 0.245 | 490 |
| 5 | 0.228 | 456 |
| Mean | 0.239 | 477.2 |
| Standard Deviation | 0.01 | 14.46 |
| CV% | 3.03 | 3.03 |

The data in the Table 3.1 indicates that the mean original concentration of fructose was 477.2 mM; the precision data of 3.3 % suggests that the method shows promise for analysis of fructose in fruit juices.

It is known that ascorbic acid is present in apple juice of concentrations around 2.15 mM [16]. In order to determine whether this vitamin would affect the response obtained for fructose, in the apple juice sample, a fixed concentration of 2.15 mM ascorbate was added to the neat apple juice followed by dilution (as described earlier). The resulting chronoamperograms did not show any increase in anodic current for any of the solutions shown in Table 1. This is perhaps not surprising bearing in mind the high ratio of fructose to ascorbic acid, present in the sample. This study demonstrated that ascorbic acid at the levels present in the commercial apple juice did not influence the magnitude of the fructose response; therefore, no complicated sample preparation procedures were required. The recovery of fructose, added to the original apple juice sample is summarised in Table 3.2.

Table 2.2. Recovery of added fructose to original fruit juice sample (n=5).

| Sample | Original Concentration (mM) | Concentration Added (mM) | Concentration Found (mM) | Fructose Recovered (mM) |
|---|---|---|---|---|
| 1 | 477.2 | 477.2 | 951.88 | 474.68 |
| 2 | 477.2 | 477.2 | 934.28 | 457.08 |
| 3 | 477.2 | 477.2 | 1010.08 | 532.88 |
| 4 | 477.2 | 477.2 | 953.79 | 476.59 |
| 5 | 477.2 | 477.2 | 992.68 | 515.48 |
| | | | Average | 491.34 |
| | | | Standard Deviation | 31.53 |
| | | | CV% | 6.42 |
| | | | Mean Recovery | 97.12 % |

The data in Table 2.2 shows that the mean recovery of added fructose is 97.12 % and the co-efficient of variation of 6.42 %. Clearly the data shown in Table 1 and Table 2.2 demonstrates that the chronoamperometric bioassay should give reliable data for the analysis of fructose concentration in fruit juice products.

## 2.4 Conclusion

This chapter describes the development of a simple, low cost chronoamperometric assay, based on the electrocatalytic oxidation of FDH with a SPCE-G-COOH, and evaluation using a commercial fruit juice. It was shown that the incorporation of modified graphite nanoparticles enhances the sensitivity and improved the precision of the response towards the enzymatically generated ferrocyanide species, compared with a plain SPCE. The calibration studies indicated that a linear response could be obtained up to 1.00 mM fructose using a mediator concentration of 3 mM. Consequently, the proposed biosensor could be adapted for a wide range of food products. It should be mentioned that sample preparation only involves the dilution of the sample prior to analysis; which is performed only on 40 µl, of solution, applied directly onto the sensor surface. Therefore, it is readily feasible that this approach could be performed near to the point of production and used as a quality control method.

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# Chapter 3

## Biosensor Construction and Investigation into Interferences

Contents – Chapter 3

## 3.1 Introduction

One issue that is problematic when using the samples from food is the interference from ascorbic acid. Ascorbic acid is a common interference that introduces errors into biosensors, designed for agri-food testing. The previous chapter showed that this interference was not detected. This was due to the apple juice having a very large ratio of fructose to ascorbic acid; therefore, the dilution factor significantly reduced the ascorbic acid concentration. Products such as potatoes have a much smaller ratio, of fructose to ascorbic acid, therefore may have an effect on the results. For example, white potatoes can typically have 0.126 mg/g and 3.599 mg/g of ascorbic acid and fructose, respectively. In comparison, apple juice can typically have 0.009 mg/g and 57.298 mg/g of ascorbic and fructose, respectively. It can clearly be seen that ratio of ascorbic acid and fructose is hugely different between white potatoes and apple juice [1]. This issue of ascorbic acid in samples also occurs in other areas such as blood and urine [2-3].

In this chapter, interference from ascorbic acid will be further explored. In addition, the conversion from a fructose bioassay to biosensor will be investigated. The reason for producing a biosensor, rather than a bioassay, is so that the end user only must introduce the test sample to the sensor. With a bioassay, the end user would have to introduce the different components to the base transducer. The bioassay could lead to increasing error factors being, as it requires more technical ability. Therefore, a biosensor is a more attractive approach, with the focus towards the end user.

## 3.2 Experimental

### 3.2.1 Chemical reagents

D-fructose dehydrogenase was obtained from Toyobo Enzymes (Japan). (www.toyobo-global.com)

The graphite-nanoparticles (graphite modified with carboxylic acid) in solution (C2131210D1) were obtained from Gwent Electronic Materials. (www.gwent.org).

All other chemicals and reagents were obtained from Sigma-Aldrich (UK). (*www.sigmaaldrich.com)*

McIlvaine buffer was prepared by mixing 0.2 M citric acid (containing 0.2 M KCl) with 0.4 M disodium phosphate (containing 0.2 M KCl) to produce a final pH of 4.5.

### 3.2.2 Apparatus and Instrumentation

All electrochemical measurements were conducted with a two-electrode system, consisting of a screen-printed working electrode (GEM code: C2030519P4), Ag/AgCl reference electrode (GEM Product Code: C61003P7) both screen-printed onto valox (a semi-crystalline material based on polybutylene terephthalate and polyethylene terephthalate polymers; Cadillac Plastics Swindon, UK). The diameter (6 mm) of the working electrode was defined using a dielectric ink (GEM Product Code: D2070423P5) a concentric silver/silver-chloride served as the counter/reference electrode, (GEM Electrode Design: BE2110916D1). For further studies, the surface of the working electrode was modified by addition of 10µl of graphite-nanoparticles (1.787 mg $ml^{-1}$) (GEM code: C2131210D1).

The working and reference electrodes were connected to the potentiostat with GEM electrode connector (GEM Code: CON002). All electrochemical studies were performed using an AutoLab [μAutoLab Type II], Metrohm, with General-Purpose Electrochemical Software (The Netherlands). Data were further analyzed with Microsoft Excel.

Fig.1 summarises the fabrication and operation of the fructose biosensor

### 3.2.3 Procedures

Cyclic voltammetry was performed by depositing a 300 μl aliquot of 0.5mM ferriycanide, in 0.1 M phosphate buffer pH 7.5 containing 0.1 M potassium chloride onto the surface of the screen-printed carbon electrodes. Cyclic voltammetry was performed using the following conditions: initial and final potential +0.8 V; switching potential –0.4 V; scan rate 10 mV $s^{-1}$. Potential held at +0.8 V for 20 seconds before initial cycle.

Studies to better understand interference by ascorbic acid were performed using chronoamperometry with standard solutions of ascorbic acid, over the concentration range 0.125 mM to 0.500 mM, made up in McIlvaine buffer. The measurement procedure involved the deposition of 20 μl of the buffer solution, onto the screen-printed transducer, followed by 10 μl of either deionized water or 12 mM ferricyanide and 10 μl ascorbic acid standard. Following an incubation time of 180 s (open circuit), with initial 20 s of agitation, the potential was stepped from open

circuit to +0.3 V vs Ag/AgCl. Currents were measured 20 s after application of the voltage and these values were used to plot calibration graphs.

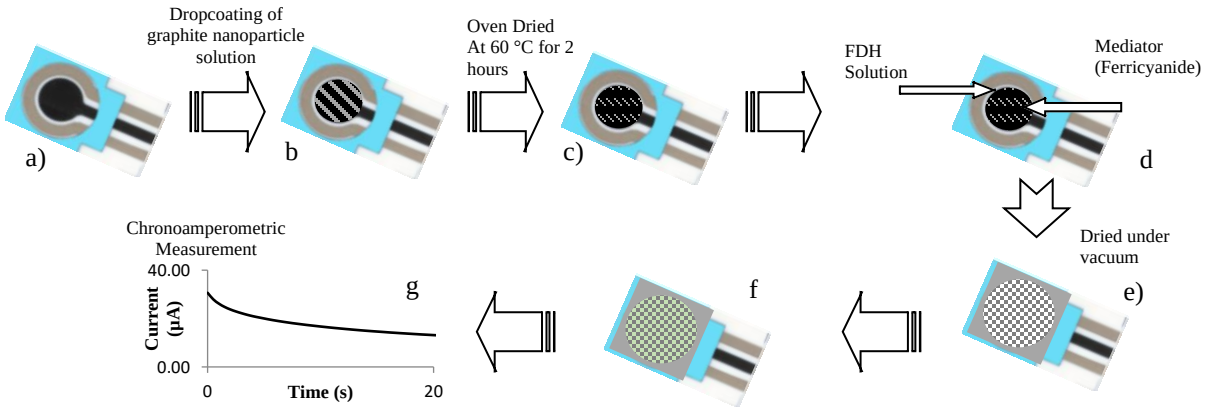



*Figure 2.1 Scheme showing the fabrication of the fructose biosensor and chronoamperometric measurement of fructose: a) Plain SPCE; b) SPCE with deposition of nanoparticles in solution; c) SPCE with dried nanoparticles; d) addition of 5 µl FDH, 5µl ferricyanide; e) The application of a well after the drying down of the FDH and ferricyanide; f) 40 µl of solution containing fructose; g) Chronoamperometric measurement*

After 5µl of FDH solution and 5µl of ferricyanide solution is dispensed onto the surface of the modified carbon electrode, the electrode is then dried under a vacuum (-800 mbar), along with fresh silica gel. This was left over night (+8 hours) to ensure that all the water was removed from the solutions, to leave a powder. A mesh well, composing of a PET carrier and adhesive with a mesh adhered on top, was placed over the electrodes. The well has two purposes, the first is to protect the components and electrode surface, during storage. The second purpose is to

provide the end user with area to pipette the solution into and agitate using a stirrer without scratching the surface of the electrode. The PET carrier has a circular cut out exposing the counter-reference and the working electrode.

## 3.3 Results and Discussion

### 3.3.1 Conversion to a biosensor

The previous chapter has described the sensor as a bioassay, as all the components are in a solution state and added to the electrode, prior to testing. The next stage involved studies involving drying the active components onto the electrode surface. The test sample (analytical fructose concentrations, 0 to 1.0 mM) was the only component that the user was required to add.

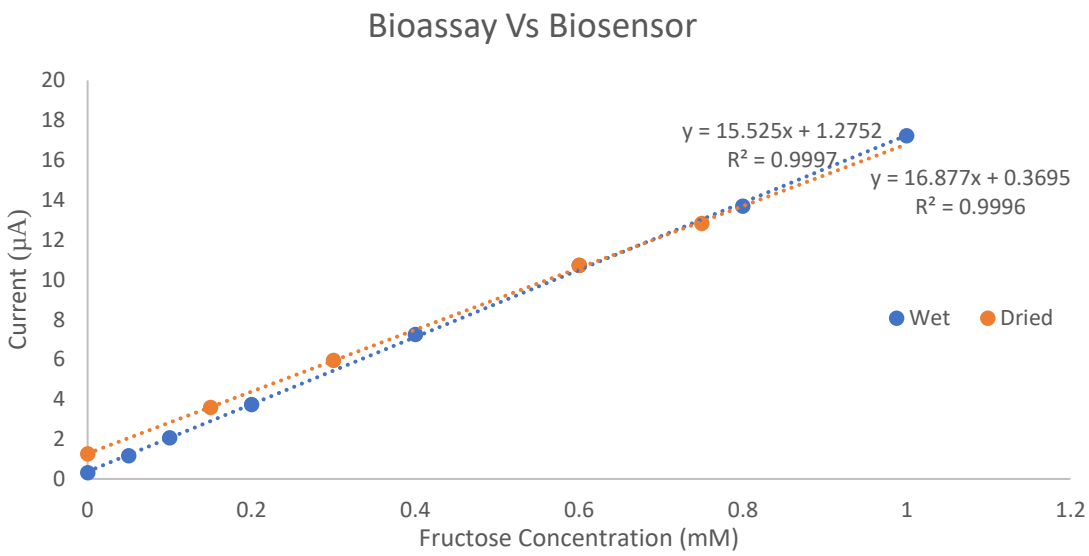



*Figure 3.2 Calibration plots obtained for fructose concentrations, comparing the bioassay and biosensor*

From the studies shown in Fig 3.2, where the FDH and mediator was dried down, there is a slight change in sensitivity but not detrimental to the end use, towards different concentrations of fructose. The difference may be due to the dried down

components not being completely dissolved into the sample solution. This may affect some of the surface area of the working electrode being available. This affects the lower concentrations as higher concentrations provides a higher exchange rate of electrons. Therefore, the system is suitable for the conversion to a biosensor, this is beneficial as it presents a system that is more desirable by the end user, in an industrial setting.

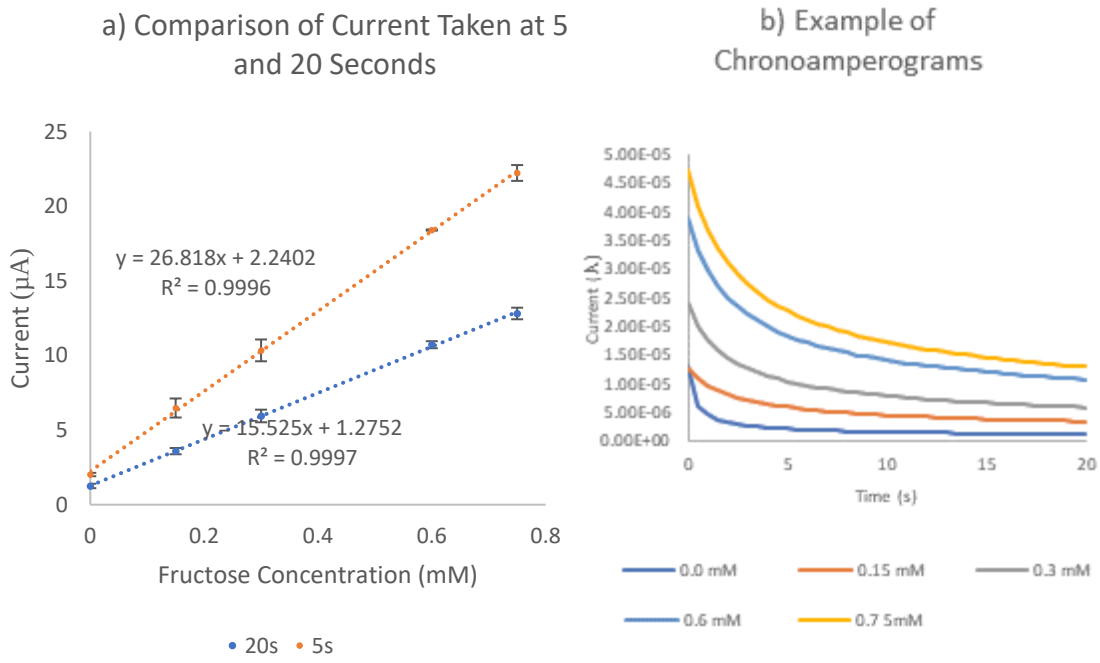



*Figure 3.3 a) Calibration plots obtained for fructose concentrations, comparing current taken at 5 seconds and 20 seconds, after an applied voltage of +0.3 V. b) examples of chronoamperograms for fructose concentrations in the range 0.00 to 0.75 mM*

In order to investigate the sensitivity of the biosensor by extracting the currents recorded, at different times of the voltage being applied. Standard solutions of fructose over the range 0.1 to 0.75 mM were measured at +0.3 V, as shown in Fig 3.3.

The measurement times were 5 seconds and 20 seconds, after applying the potential. The gradient increased from 15.525 µA/mM to 26.818 µA/mM, therefore increasing the sensitivity from 59.45 µA $mM^{-1}$ $cm^{-2}$ to 102.82 µA $mM^{-1}$ $cm^{-2}$.

From the graph, the standard deviation (error bars) are larger with the 5s but when the co-efficient of variation (CV%) is compared, the CV% for 5 seconds on average has a lower value, as shown in Table 3.1.

Table 3.1

| Fructose Concentration (mM) | CV% (n=5) | |
|---|---|---|
| | 20 s | 5 s |
| 0.00 | 11.896 | 5.581 |
| 0.15 | 5.852 | 9.843 |
| 0.30 | 7.113 | 7.062 |
| 0.60 | 2.206 | 0.206 |
| 0.75 | 3.031 | 2.384 |

### 3.3.2 Further Investigation into Ascorbic Acid Interference

In the previous chapter, ascorbic acid was at very low concentrations relative to the fructose concentration. Therefore, the electrochemical signal produced from the ascorbic acid was not detected. However, in produce such as potatoes for crisping, the ascorbic acid may produce an electrochemical signal [4-6]. To see whether the ascorbic acid is producing a signal at the running potential of the biosensor the base transducer was tested against a series of ascorbic acid concentrations. The ascorbic acid concentrations were also carried out along with the mediator, this was to find whether the mediator has any impact.

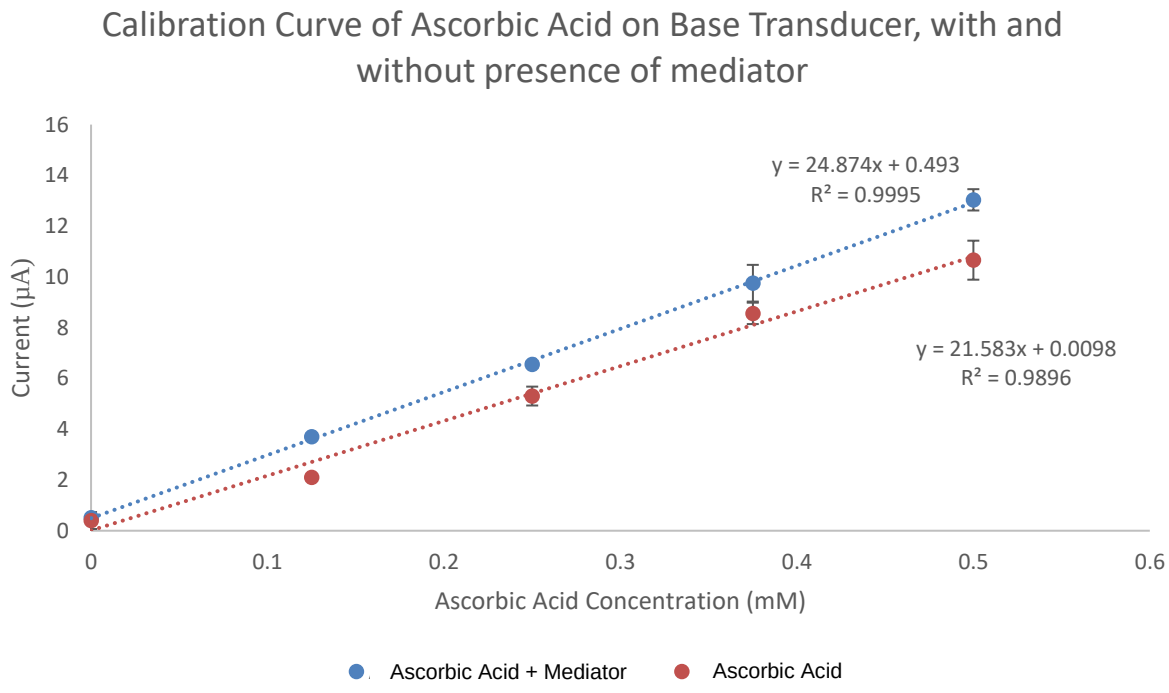



Figure 3.4 *Calibration plots obtained for ascorbic concentrations, comparing the presence and absence of ferricyanide.*

From the results obtained in Fig 3.4, ascorbic acid presents an electrochemical signal, with the base transducer at +0.3 V. Therefore, it can be considered that there is a possibility that relative concentration levels of ascorbic acid (compared to the fructose concentration) will have an accumulative effect to the signal produced. In addition, the current produced from the ascorbic acid is increased in the presence of the mediator (ferricyanide).

To investigative the reason for the increased signal, in the presence of the mediator, a series of voltammograms where carried out, comparing the current being produced at different voltages for ascorbic acid and ferricyanide.

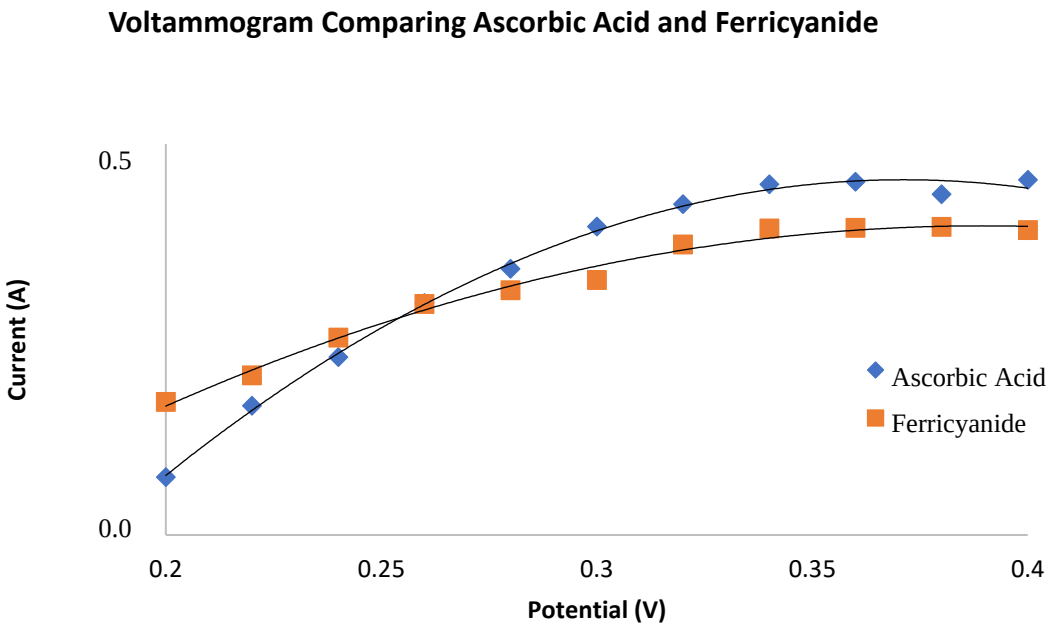



*Figure 4.5 Voltammograms comparing ascorbic acid and ferrocyanide.*

From the voltammograms produced in Fig 4.5, the plateau of ferricyanide occurs at a lower potential than that of ascorbic acid. When a potential of +0.3V is applied the reduced ferricyanide (ferrocyanide), at the Nernst layer is oxidised. In contrast, the ascorbic acid present at the Nernst layer is not completely oxidised, at +0.3 V. The ascorbic acid requires a potential closer to +0.35 V.

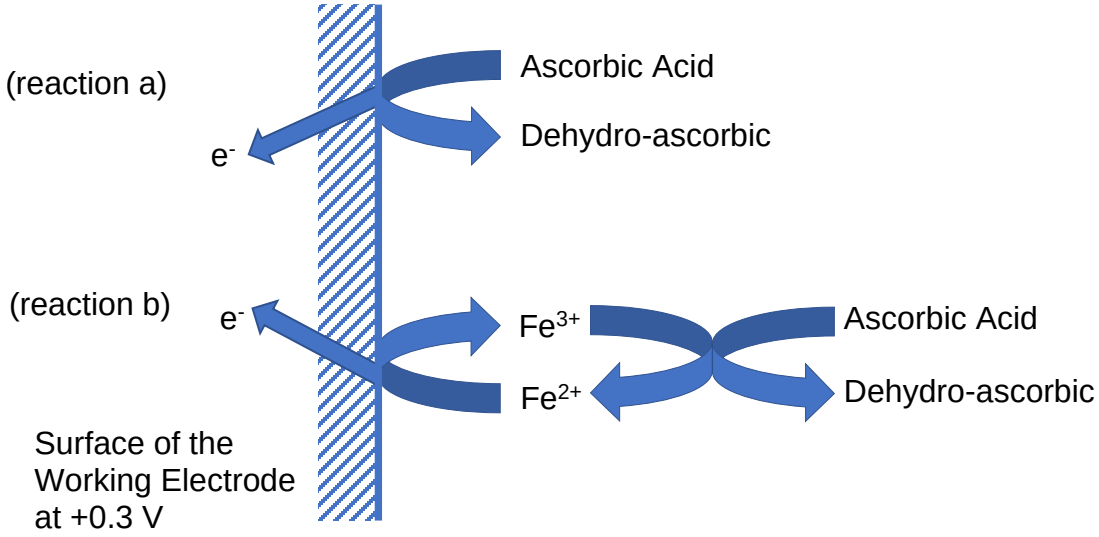



*Figure 3.6 Proposed mechanism for the difference in electrochemical signal of ascorbic acid in the presence of ferricyanide.*

a) Mechanism of Ascorbic Acid reacting directly with the working electrode

b) Mechanism of Ascorbic Acid reducing the ferricyanide (represented by $Fe^{2+}$) to ferrocyanide (represented by $Fe^{2+}$). The ferrocyanide is then oxidised back to ferricyanide by the working electrode

Fig 3.6 proposes that at +0.3 V potential, the ascorbic acid can become directly oxidised to dehydro-ascorbic acid by the working electrode. However, the potential isn't positive enough to convert all the ascorbic acid molecules, at the Nernst layer. When ferricyanide is present, the ascorbic acid becomes oxidised by the ferricyanide. In turn, the ferricyanide become reduced to ferrocyanide. The ferrocyanide molecules are easily oxidised back to ferricyanide, at the Nernst layer, at a potential of +0.3 V. Therefore, the number of electrons accepted by the working electrode is higher when ferricyanide is present, resulting in higher current being produced.

### 3.3.3 Analytical studies

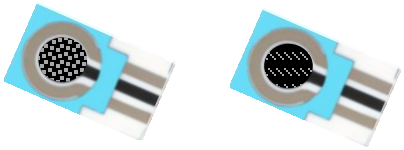
#### 3.3.3.1 Initial Studies of Linear Substitution Approach

An attractive approach of removing the ascorbic acid interference (and possibly other interferences at +0.3 V) is via linear substitution [7]. This was achieved with the use of two sensors/biosensors. One sensor will be constructed with mediator and buffer (referred to the background sensor) and the other constructed with the mediator, buffer and fructose dehydrogenase (referred to as the fructose biosensor).

The current produced by the background sensor will be subtracted from the current produced by the fructose biosensor. The resulting current will represent the current being produced from the presence of fructose.

For the linear substitution to work, the base transducer for both the background sensor and fructose biosensor were constructed from the same materials and designs. This ensures that the interaction on the working electrode is the same and the area of the electrodes are consistent.

Background Sensor, constructed with buffer and mediator



Fructose Biosensor, constructed with mediator, buffer and fructose dehydrogenase

*Figure 3.7 Diagrammatic Summary of the Background Sensor and the Fructose Biosensor.*

Before using the background sensor, three solutions were tested on the fructose biosensor. This was to confirm that the background ascorbic acid can be removed by linear substitution. The resulting currents were analysed using the following equation:

$$\frac{i_{F+AA} - i_{AA}}{i_F} = 1$$

$i_{F+AA}$ = current of fructose and ascorbic acid solution

$i_{AA}$ = current of ascorbic acid solution

$i_F$ = current of fructose solution

Theoretically, the current of fructose solution of [A] mM concentration should be the same as the current of fructose and ascorbic acid of [A] mM and [B] mM respectively minus the current of ascorbic acid of [B] mM. Thus, the two values should be the same. To quantify any variation, ($i_{F+AA}$ - $i_{AA}$) can be divided by $i_F$ and any difference from the value of 1 can be observed.

Results from the initial linear substitution test.

$$\frac{i_{F+AA} - i_{AA}}{i_F} = \frac{41.09 - 21.16}{21.03} = 0.95$$

From the results, there is a 5% discrepancy. However, the test was comprised from three separate analytical solutions (containing different combinations of fructose and ascorbic acid). This was considered a good starting result and further testing with the two sensors was required.

### 3.3.3.2 Further Studies of Linear Substitution Approach

To test whether the use of the background sensor is a suitable approach, a recovery procedure was carried out to see if added ascorbic acid could be recovered using potato juice fortified with ascorbic acid and without. The currents produced was analysed using the following equation:

$$\frac{i_{FDH\ Biosensor\ (Spiked\ Sample)} - i_{FDH\ Biosensor\ (Sample)}}{i_{Background\ Sensor\ (Spiked\ Sample)} - i_{Background\ Sensor\ (Sample)}} = 1$$

$i_{FDH\ Sensor\ (Spiked\ Sample)}$ = Current of potato juice spiked with ascorbic acid and tested on the Fructose Biosensor

$i_{FDH\ Sensor\ (Sample)}$ = Current of potato juice tested on the Fructose Biosensor

$i_{Background\ Sensor\ (Spiked\ Sample)}$ = Current of potato juice spiked with ascorbic acid and tested on the Background Sensor

$i_{Background\ Sensor\ (Sample)}$ = Current of potato juice tested on the Background Sensor

Sample of potato juice was obtained and tested using the fructose sensor and also the background sensor. The same potato juice was spiked with 0.1 mM ascorbic acid and then retested using the fructose sensor and the background sensor

$$\frac{18.34\ \mu A - 15.13 \mu A}{3.57 \mu A - 0.45 \mu A} = \frac{3.21 \mu A}{3.12 \mu A} = 1.03$$

From the results, the ascorbic acid was recovered with a 3% error. This showed that the approach of a two sensor/biosensor system for linear substitution is feasible

### 3.3.4 Standard Addition of Fructose to Potato Juice

In order to evaluate the chronoamperometric assay for the measurement of fructose in extracted potato juice, a standard addition assay was produced, as shown in Fig 3.8.

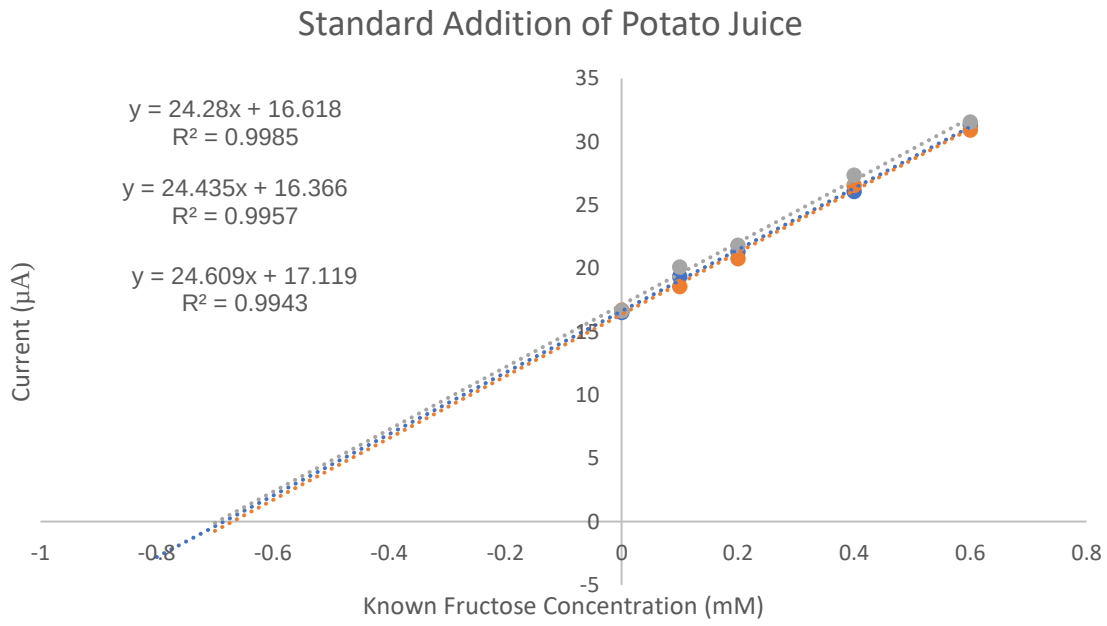



*Figure 3.8. Three typical standard addition plot, obtained using chronoamperometric currents measured 20 s after application of +0.3 V vs Ag/AgCl.*

Table 3.2

| Sample | Concentration determined after dilution (mM) | Calculated Original Fructose Concentration (mM) |
|---|---|---|
| 1 | 0.68443 | 2.73773 |
| 2 | 0.69564 | 2.78256 |
| 3 | 0.66978 | 2.67911 |
| Mean | 0.68328 | 2.73313 |
| Standard Deviation | 0.01297 | 0.05188 |
| CV% | 1.89814 | 1.89814 |

Table 3.2 shows a summary of the data obtained for the original fructose concentration of the potato juice extract. It should be noted that column 2 refers to the concentration on the screen-printed transducer; column 3 refers to the concentration of fructose corrected for the dilution factors.

3.3.5 Recovery of Fructose from Potato Juice Extract

Table 3.3

| Sample | Original Concentration (mM) | Concentration Added (mM) | Concentration Found (mM) | Fructose Recovered (mM) |
|---|---|---|---|---|
| 1 | 2.733 | 2.733 | 5.448929 | 2.715809 |
| 2 | 2.733 | 2.733 | 5.605931 | 2.872811 |
| 3 | 2.733 | 2.733 | 5.532949 | 2.799829 |
| 4 | 2.733 | 2.733 | 5.465568 | 2.732448 |
| 5 | 2.733 | 2.733 | 5.409555 | 2.676435 |
| | | | Average | 2.759 |
| | | | Standard Deviation | 0.77 |
| | | | CV% | 2.807 |
| | | | Mean Recovery | 100.96 % |

The data in Table 3.3 shows that the mean recovery of added fructose is 100.96 % and the co-efficient of variation of 2.807 %. Clearly the data shown in Table 3.2 and Table 3.3 demonstrates that the chronoamperometric biosensor should give reliable data for the analysis of fructose concentration in low level sugar potatoes.

## 3.4 Conclusion

This chapter describes the conversion of the fructose chronoamperometric assay to a biosensor system, with minimal impact to the overall functionality. The system has been adapted to analyse samples containing higher ascorbic acids concentration closer to the concentration of fructose. The use of a linear subtraction two sensor/biosensor system eliminates any electrochemical signal produced from interference molecules, such as ascorbic acid and any unforeseeable interferences.

In addition, the sensitivity of the system has been found to be improved by using the chronoamperometric current measured at 5 seconds, instead of 20 seconds. This resulted in the increased the sensitivity from 15.525 μA/mM to 26.818 μA/mM.

The proposed biosensor system has been found to be suitable for food products, such as industrial potatoes for crisp manufacturing. Therefore, it is readily feasible that this approach could be performed near to the point of production and used as a quality control method.

## 3.5 References

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# Chapter 4

# Development of Low Concentration Glucose and Sucrose Biosensors

Contents – Chapter 4

## 4.1 Introduction

### 4.1.1 Acrylamide in food production

The requirement of measuring glucose and sucrose has become increasingly important to the food industry (e.g. crisps), this is owing to the discolouration of the product by reducing sugars, this problem leads to the loss of the product. When potatoes are processed into fried products (such as crisp snacks and chipped potato), the concentration of glucose corresponds to the appearance of the discoloration of the product. The more of the reducing sugar (glucose), the darker the product [1-5].

The sugar sucrose is found in higher concentrations, in immature potatoes [6]. The potato uses the sugar for the synthesis of starch, resulting in growth of the plant. In storage conditions, the enzyme invertase becomes active, converting sucrose to the reducing sugars (fructose and glucose), leading to the undesirable discolouration during the processing.

In November of 2017, the European Union introduced a new commission regulation, regarding acrylamide. It relates to the control measures and the reduction of acrylamide, in food. Acrylamide is regarded as a chemical hazard, produced by reducing sugars and asparagine at temperatures about 120°C, in the presence of low moisture. For food such as potato crisps, from fresh potatoes, the benchmark level is 750 µg/kg of acrylamide [7].

4.1.2 The Maillard Reaction

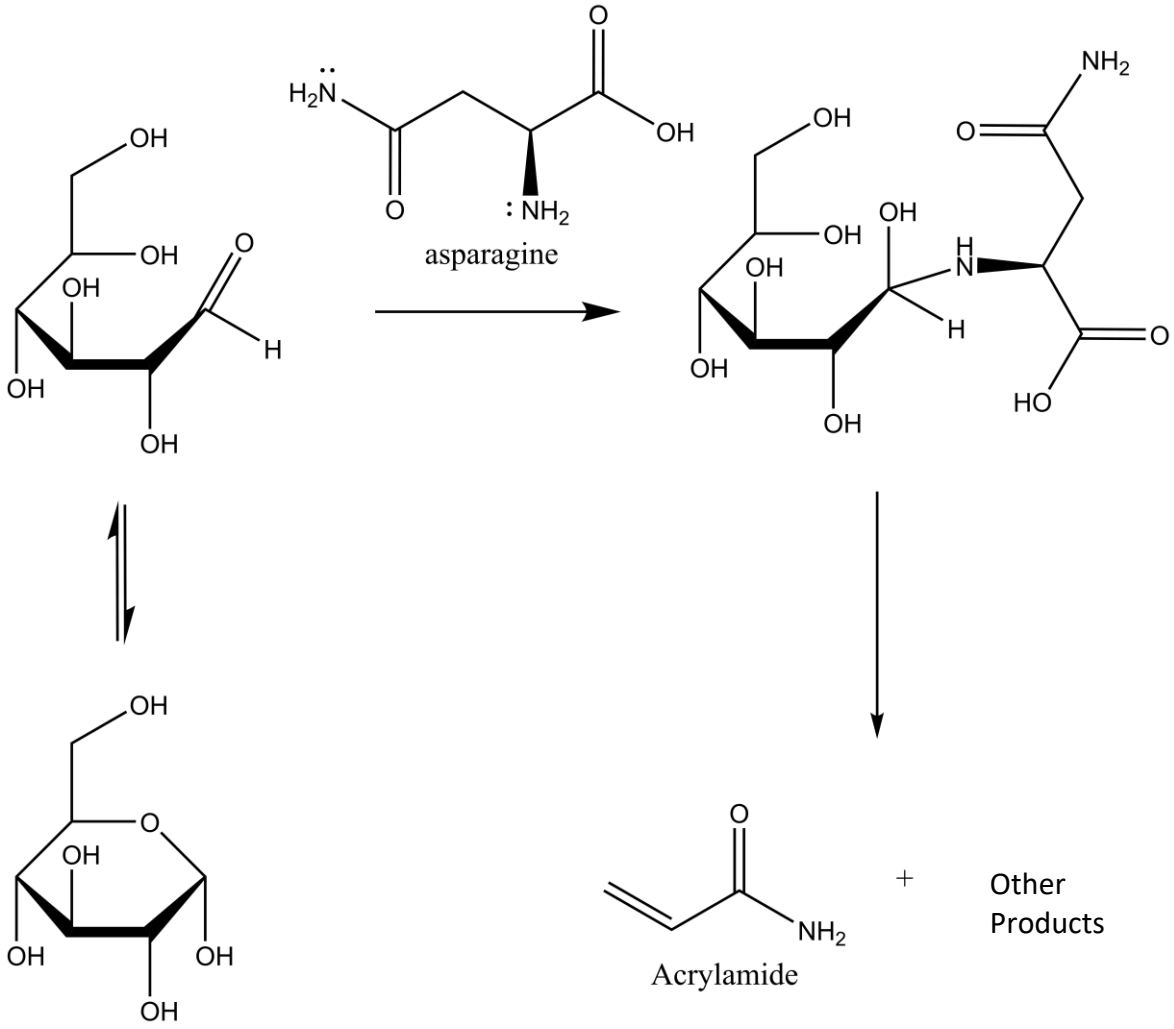



*Figure 4.1 The Chemical Reaction of the Maillard Reaction*

Fig 4.1 shows the Maillard reaction, it is a series of reactions that occur between reducing sugars and amino acids/peptides [8]. During the production of frying of food, reducing sugars such as glucose and the amino acid asparagine undergo this reaction. The leads to the browning (or caramelisation) of the food. As a result of this the chemical acrylamide is produced. Many factors can influence the speed of the reaction, including pH, temperature, etc [8].

The measurement of acrylamide, in the final product, is not useful for the industry. Instead, measuring and monitoring of the precursors of acrylamide is more appropriate, as this can then be controlled before the production of the final product.

The most common reducing sugars present in food are glucose and fructose [9]. Consequently, the accurate measurement of these sugars is essential in eliminating the amount of acrylamide produced. This chapter will describe the development of an electrochemical biosensor for the determination of sucrose and glucose.

### 4.1.3 Glucose Biosensors

As discussed in Chapter 1, there have been developments of glucose since 1962 by Clark and Lyons [10-11].

Currently on the market for the measurement of glucose in crops, there is the Gluco-LIS Blue, by Martin Lishman [12]. This technology is based on commercially available blood glucose biosensors. This technology is only practical with produce containing higher levels of glucose (and does not report sucrose). This cannot measure the glucose food products, such as processing potatoes (a type of potato

cultivated to have low sugar content) as the concentrations are below the limit of detection of this device, 1.80 mM.

Another commercially available system, to measure glucose and sucrose, is manufactured by Yellow Springs Instruments (YSI) [13]. This uses YSI 2900 Series Biochemistry Analyzer - equipped with a specialised Membrane, containing impregnated glucose oxidase. The system is based on electrochemical measurements. However, it is unable to measure the lower concentrations of glucose and sucrose, required by the food industry.

The approach for this system was designed in a different matter. The system utilises the combination of glucose oxidase, hydrogen peroxidase and a ferrocene-based mediator. The reason for this was to avoid the introduction of a “interference” sensor, such as the proposed fructose sensor. The use of a ferrocene-based mediator allows the chronoamperometric measurements to be made in the reduction phase and measured at a low negative potential. This approach doesn’t require an “interference” sensor, as there are very few interferences that are measured in the reduction phase and a low negative potential. The fructose sensor(s) were not able to use this method, due to the enzyme being a dehydrogenase, thus not interacting with hydrogen peroxidase (which then interacts with the ferrocene-based mediators).

## 4.2 Experimental

### 4.2.1 Chemical reagents

Mutoratose, glucose oxidase and horseradish peroxidase were obtained from BBI Solutions. (www.bbisolutions.com)

The ferrocene-based mediator ($FCR_2$) was obtained from Gwent Electronic Materials, Pontypool, UK. (www.gwent.org).

All other chemicals and reagents were obtained from Sigma-Aldrich (UK). (*www.sigmaaldrich.com)*

### 4.2.2 Apparatus and Instrumentation

All electrochemical measurements were conducted with a two-electrode system, consisting of a screen-printed working electrode (GEM code: C2030519P4), Ag/AgCl reference electrode (GEM Product Code: C61003P7) both screen-printed onto valox (a semi-crystalline material based on polybutylene terephthalate and polyethylene terephthalate polymers; Cadillac Plastics Swindon, UK). The diameter (6 mm) of the working electrode was defined using a dielectric ink (GEM Product Code: D2070423P5) a concentric silver/silver-chloride served as the counter/reference electrode, (GEM Electrode Design: BE2110916D1).

The working and reference electrodes were connected to the potentiostat with GEM electrode connector (GEM Code: CON002). All electrochemical studies were performed using an AutoLab [μAutoLab Type II], Metrohm with General-Purpose

Electrochemical Software (The Netherlands). Data were further analyzed with Microsoft Excel.

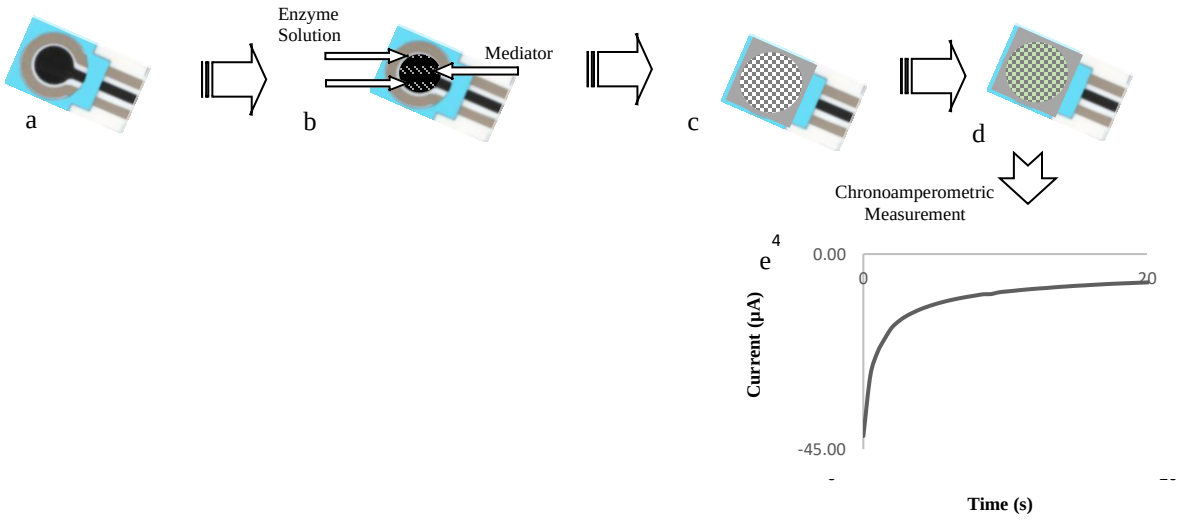



*Figure 4.3. Scheme showing the fabrication of the biosensors and chronoamperometric measurement of glucose: a) Plain SPCE; b) Dispensing of 5 µl of enzyme solution and 5 µl enzyme solution (mixture of glucose oxidase, horseradish peroxidase and mutarotase). The solutions were then dried down under a vacuum of -800 mbar; c) A well was placed on top of the dried sensor; d) 40 µl of test solution deposited onto the sensor; e) Chronoamperometric measurement*

### 4.2.3 Procedures

Cyclic voltammetry was performed by depositing a 40 µl aliquot of 0.5mM ferrocene-based mediator, in 0.1 M phosphate buffer pH 7.5 containing 0.1 M

potassium chloride onto the surface of the screen-printed carbon electrodes. Cyclic voltammetry was performed using the above parameters.

Further cyclic voltammetry for comparison of ferrocene-based mediators was performed by depositing a 40 µl aliquot of 0.5 mM mediator, 1 mM glucose and 4 U of glucose oxidase, in 0.1 M phosphate buffer pH 7.5 containing 0.1 M potassium chloride onto the surface of the screen-printed carbon electrodes. Cyclic voltammetry was performed using the following conditions: initial and final potential -0.15 V; switching potential +0.4 V; scan rate 10 mV $s^{-1}$.

Calibration studies were performed using chronoamperometry with standard solutions of glucose or sucrose, over the concentration range 0.025 mM to 1.000 mM, in water; enzymes were dissolved in 0.4 mM phosphate buffer. The measurement procedure involved the deposition of 5 µl of either enzyme solution, onto the screen-printed transducer, followed by 5 µl of mediator solution and 30 µl fructose standard. Following an incubation time of 180 s (open circuit), with initial 20 s of agitation, the potential was stepped from open circuit to -0.2 V vs Ag/AgCl. Currents were measured 20 s after application of the voltage and these values were used to plot calibration graphs.

For biosensor studies, the ferrocene-based mediator and enzyme solution, containing glucose oxidase, horse radish peroxidase and mutarotase, were deposited as two 5µl individual spots. The sensors were then dried with negative

pressure of -800 mbar, for a minimum of eight hours. A 40 µl aliquot of standard (glucose or sucrose) was deposited onto the sensor. Following an incubation time of 180 s (open circuit), with initial 20 s of agitation, the potential was stepped from open circuit to -0.2 V vs Ag/AgCl. Currents were measured 20 s after application of the voltage and these values were used to plot calibration graphs.

4.2.4 Analytical Application – Glucose Biosensor

The sequence of the reactions involved in the operation of the biosensor are shown in Fig 4.5

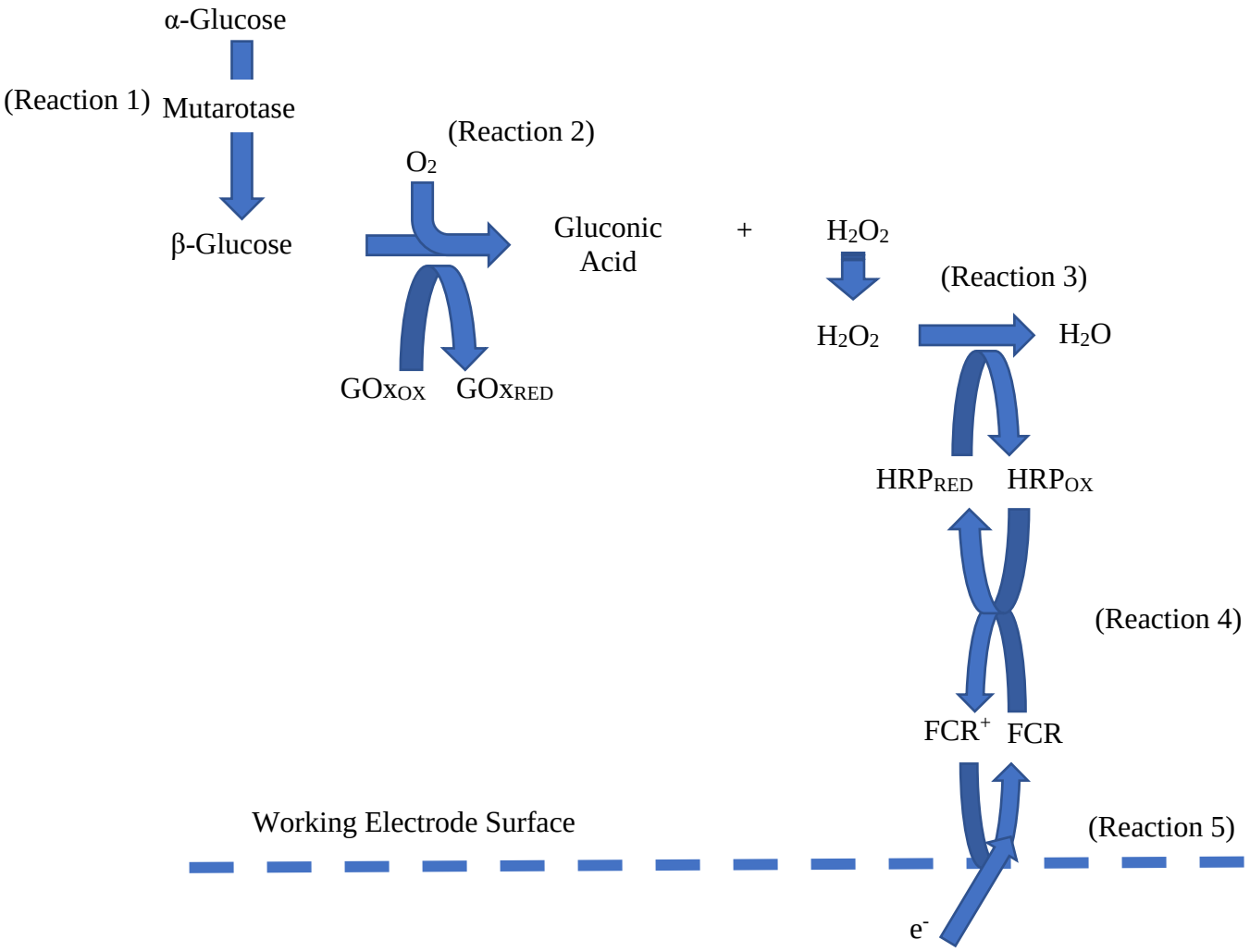



Figure 4.5 The proposed mechanism for the glucose sensor

Reactions:

1) α-Glucose + Mutarotase ⇋ β-Glucose + Mutarotase
2) β-Glucose + $O_2$ + Glucose Oxidase$_{(Ox)}$ → Gluconic Acid + $H_2O_2$ + Glucose Oxidase$_{(Red)}$
3) $H_2O_2$ + Horseradish Peroxidase$_{(Red)}$ → $H_2O$ + Horseradish Peroxidase$_{(Ox)}$
4) Horseradish Peroxidase$_{(Ox)}$ + FCR → Horseradish Peroxidase$_{(Red)}$ + $FCR^+$
5) $FCR^+ + e^- \rightarrow FCR$

Reaction 5 only occurs when the potential of -0.2V is applied and is responsible for the analytical response

The following section describe the biosensor components together with the biochemical and electrochemical processes which occur during the operation of the biosensor.

4.2.4.1 Introduction of Mutarotase

Glucose is found in various isomers, in nature, the most common isomers are α-D-glucose and β-D-glucose [14], as shown in Figure 4.6.

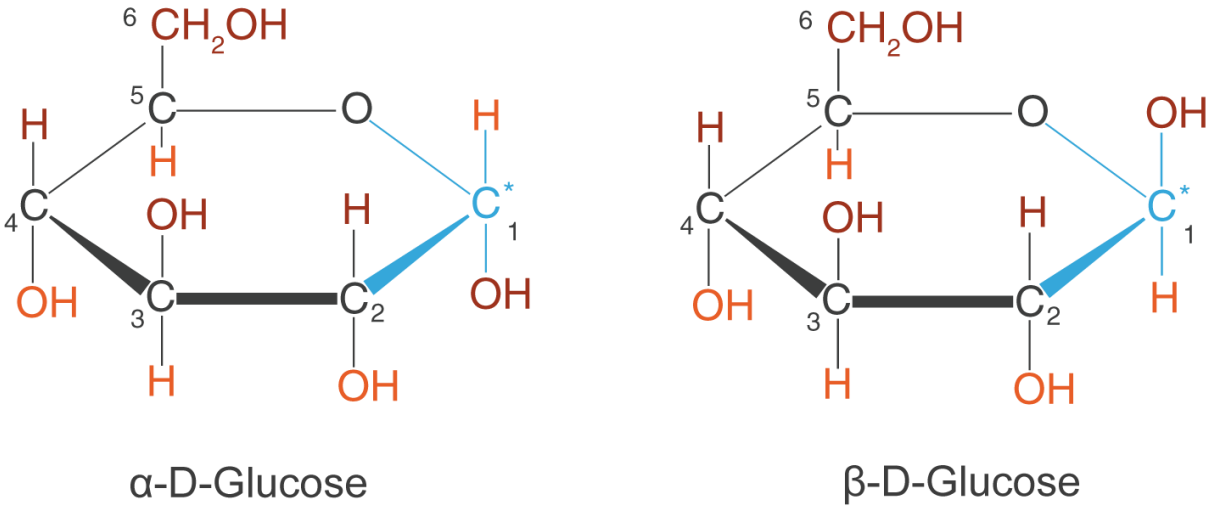



Figure 4.6 Chemical Structures of D-Glucose isomers

Glucose Oxidase converts the β-D-glucose form to gluconic acid and hydrogen peroxide, under aerobic conditions. The ratio of the alpha form to the beta form is approximately 1:2, respectively, when dissolved in water [15].

To measure all the glucose present, in the solution, the enzyme mutarotase (aldose-1-epimerase) was added. This converts the α-D-glucose into the β-D-glucose, which allows all the present glucose to be converted by the glucose oxidase.


*Figure 4.7 represents the enzymatic reaction: α-Glucose + Mutarotase ⇋ β-Glucose + Mutarotase*

To ensure that the isomers of glucose (α - glucose and β - glucose) are measured, mutarotase was added. To deduce whether it had an affect on the operation of the biosensor

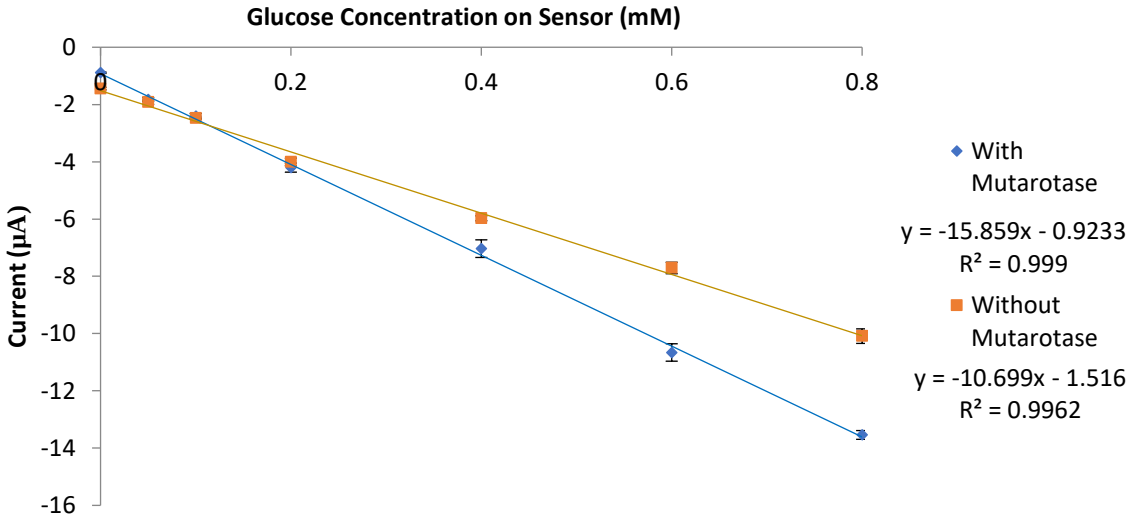



*Figure 4.8 Graph showing the difference between the inclusion and exclusion of mutarotase*

Clearly from Fig 4.8 the biosensors containing the mutarotase produced an elevated response. Therefore, this enzyme is required to measure the α and β forms of the glucose, producing a measurement of the total concentration of glucose and contributing to the analytical response.

4.2.4.2 Limitation of system based on Oxygen

One of the issues of using the enzyme glucose oxidase is that oxygen is required, in the proposed biosensor. This causes the system to be limited by the oxygen concentration in the test sample.

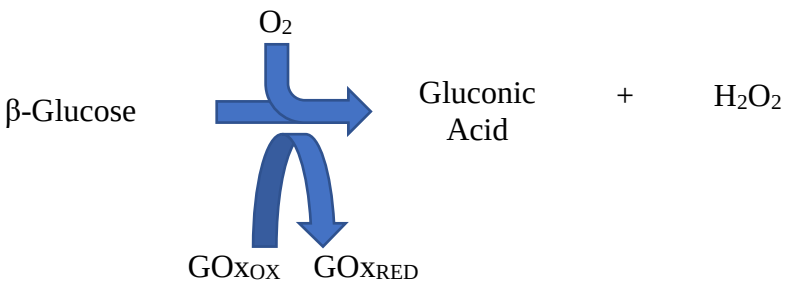



*Figure 4.9 represents the enzymatic reaction: β-Glucose + $O_2$ + Glucose Oxidase$_{(Ox)}$ → Gluconic Acid + $H_2O_2$ + Glucose Oxidase$_{(Red)}$*

A calibration study was carried out to investigate the impact of oxygen concentration in the sample, to the system. When samples are taken from produce, such as potato juice extract, the oxygen concentration should be higher. This is due to the native oxygen that is released from the produce, prior to juicing.

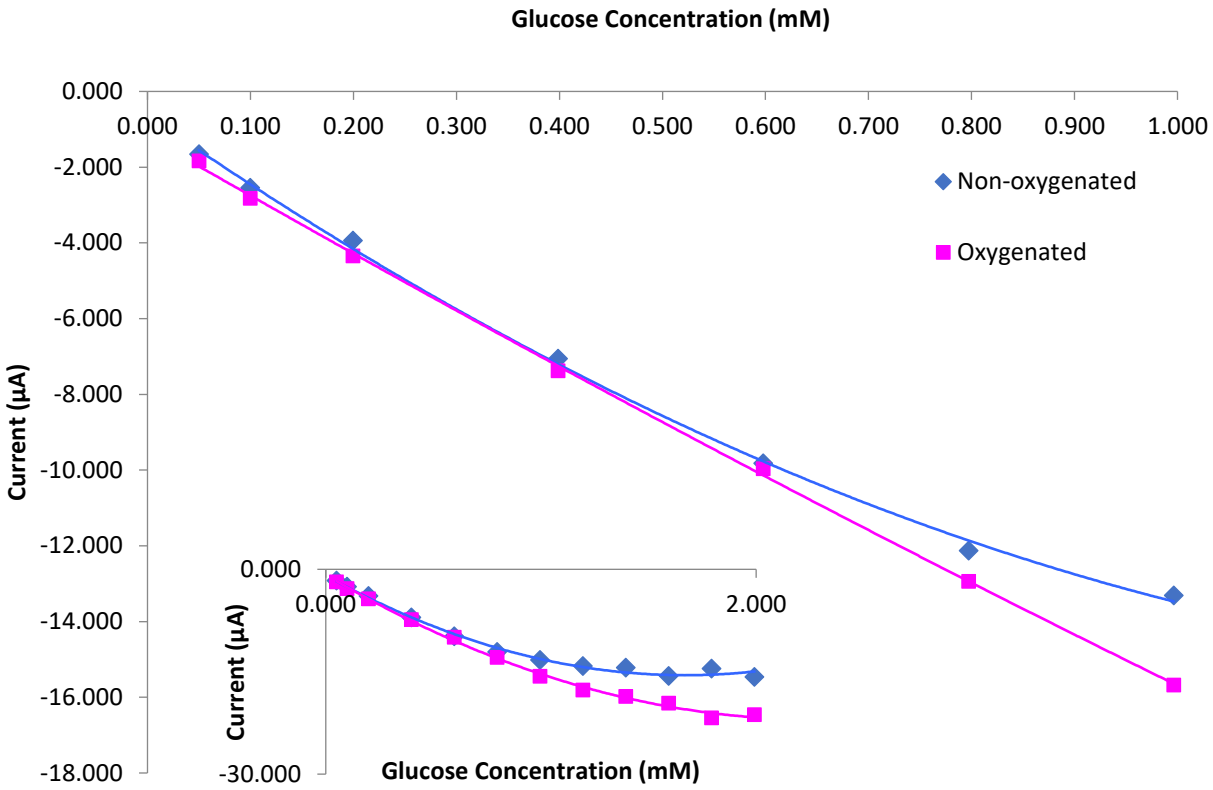
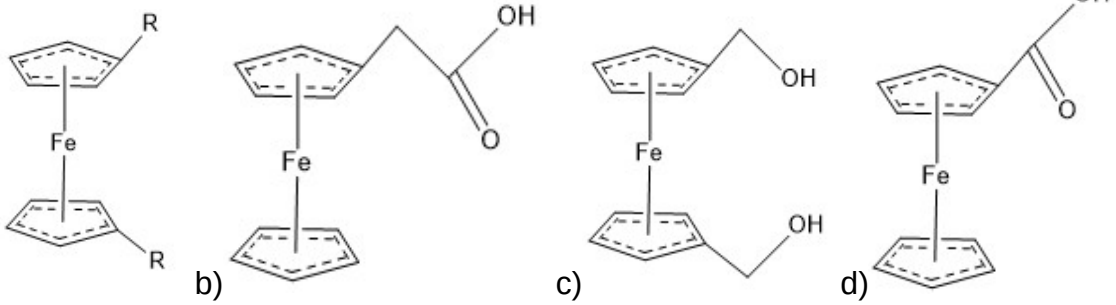



*Figure 4.10 demonstrates that the system is oxygen dependent. As the end user will not be oxygenating the test solution before use, the maximum that can be monitored is 0.6 mM. This can be easily observed when the graph is extended to 2.0 mM.*

4.2.4.3 Comparison of Different Ferrocenes as Potential Mediators

Cyclic voltammetry was used to investigate the electrochemical behaviour of four ferrocene compounds that were considered as mediators for the proposed glucose biosensor.

The choice of mediator was suggested for review from Gwent Electronic Materials, it was a ferrocene-based molecule (referred to as Fc-$R_2$) that was synthesised by the company. Three other meditators were compared with the Fc-$R_2$, the three were also ferrocene derivatives. In total the following were tested: Fc-$R_2$ (labelled a), the R-group is subject to intellectual property and so cannot be specified; ferrocene ethanoic acid (labelled b); 1-1' ferrocene bismethanol (labelled c) and ferrocene carboxylic acid (labelled d). *Only three types of ferrocene-based mediators were tested due to limited availability during time of testing.*



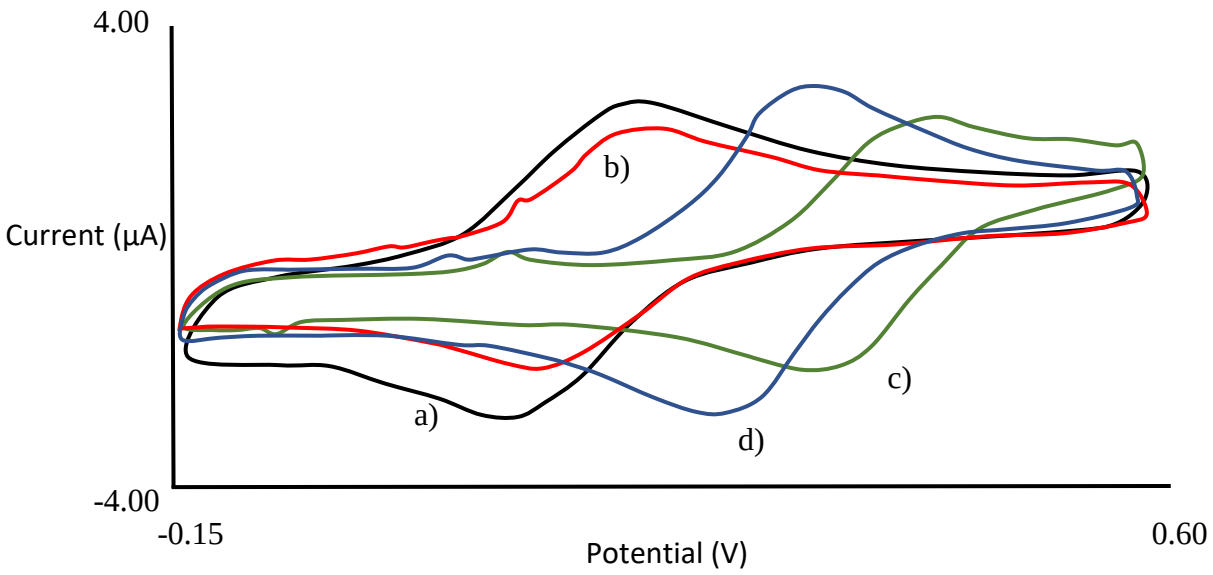



*Figure 4.11 Comparison of the cyclic voltammetric behaviours for the four ferrocene compounds.*

a) Fc-$R_2$

b) Fc-$CH_2CO_2H$ (ferrocene ethanoic acid)

c) Fc-$(CH_2OH)_2$ (1-1' ferrocene bismethanol)

d) Fc-$CO_2H$ (ferrocene carboxylic acid)

The cyclic voltammetograms, from Fig 4.11, appear to show reversible behaviour for the four ferrocene derivatives. Therefore, at this stage there is no obvious advantage of using one compound from another.

When cyclic voltammograms are undertake with the mediators, in the presence of GOx and glucose, only the $FCR_2$ mediator retains its reversible behaviour, as it does not interact with glucose oxidase.

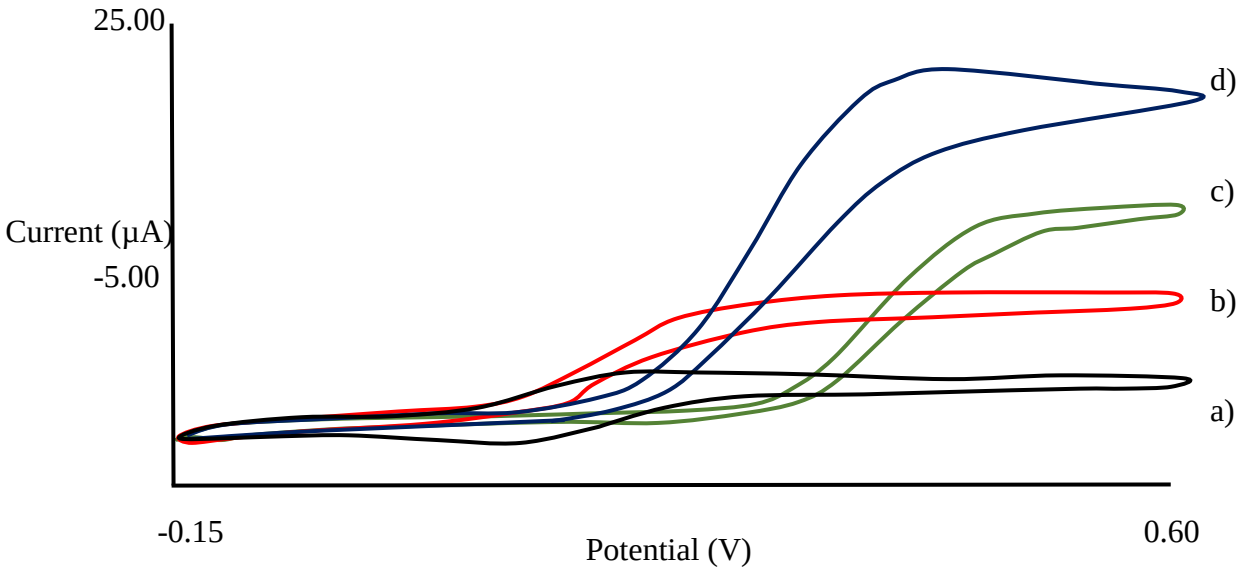



*Figure 4.12 Cyclic Voltammograms of different ferrocene-based mediators in the presence for glucose oxidase and glucose.*

a) $FcR_2$

b) Fc-$CH_2CO_2H$ (ferrocene ethanoic acid)

c) Fc-$(CH_2OH)_2$ (1-1' ferrocene bismethanol)

d) Fc-$CO_2H$ (ferrocene carboxylic acid)

The derivatives b,c and d do not show reversible behaviour. The oxidative state is present but the reduction state is lost. The $FcR_2$ retains the reversable behaviour, retaining both oxidative and reduction states. The reason for this can be explained as follows.

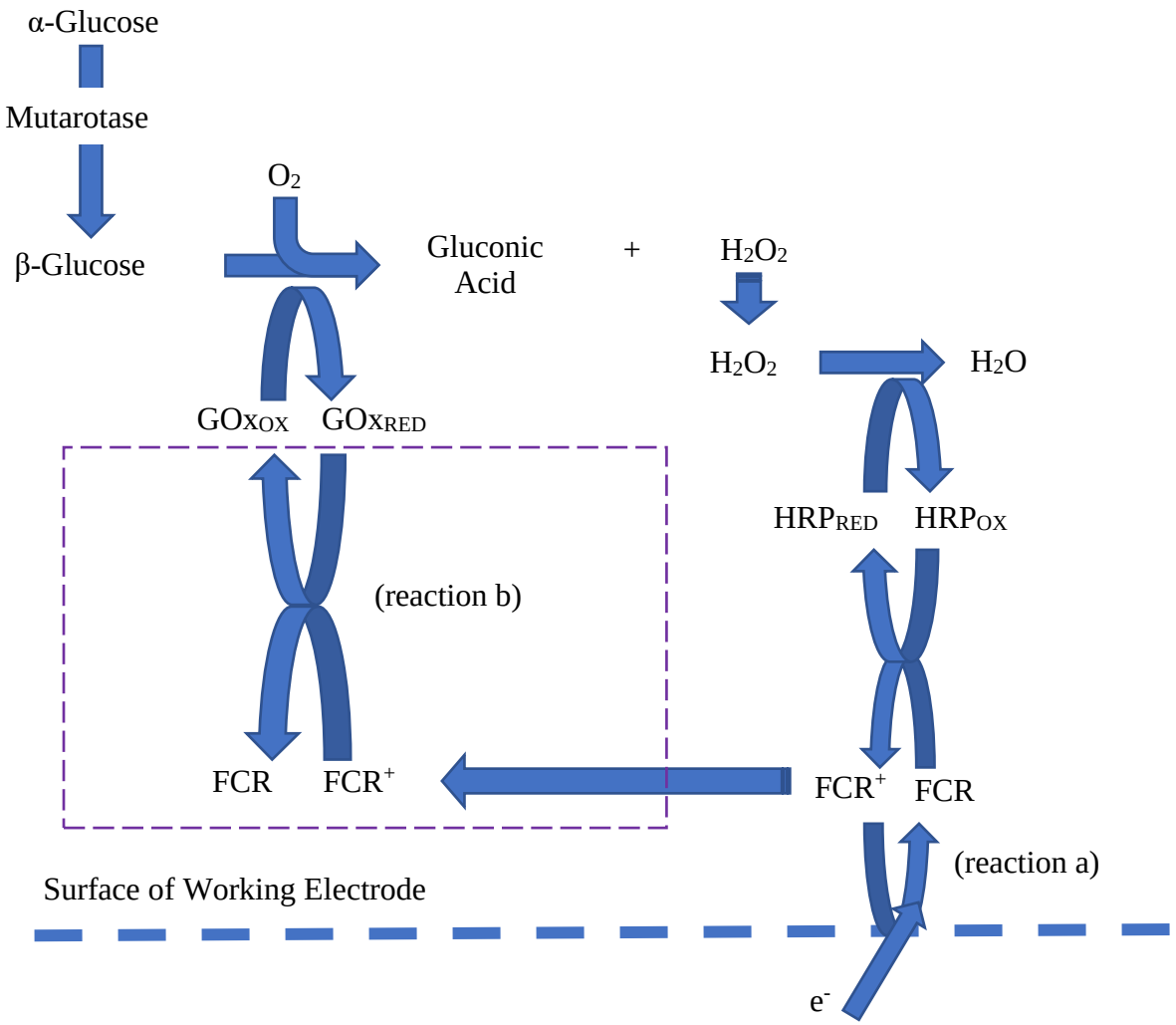



*Figure 4.13 Competition Mechanism with Different Ferrocenes*

a) $FCR^+ + e^- \rightarrow FCR$

b) $FCR^+ + \text{Glucose Oxidase}_{(Red)} \rightarrow FCR + \text{Glucose Oxidase}_{(Ox)}$

The anodic potential is “lost”, when a cyclic voltammogram is performed. This is due to the $FCR^+$ generated is reduced by the glucose oxidase and *not* by the surface of the working electrode. There is competition between the working electrode and the reduced glucose oxidase. This only occurs at small concentrations of glucose, as the generated $FCR^+$ is limited and reduced by the

glucose oxidase. This results in the working electrode not donating electrons and so no (or little) current is observed. At larger concentrations of glucose, the concentration of generated $FCR^+$ is high enough to interact with the reduced glucose oxidase, and the working electrode, producing a current

For the $FCR_2$, this interaction doesn't occur between the $FCR^+$ and the reduced glucose oxidase. This results in the $FCR^+$ only being reduced by the surface of the working electrode, when anodic potential is applied.

The lack of interaction between the $FCR^+$ and the glucose oxidase, in the presence of glucose, is advantageous when measuring low concentrations of glucose. It should be mentioned that the YSI, and other devices, cannot operate at the low concentrations (below 0.100 mM) possible with the proposed biosensor.

#### 4.2.4.4 Ensuring Sufficient Time for End-Point Reaction to Occur

A test was carried out to check whether 3 minutes was an appropriate time, for an endpoint to be achieved, where all the glucose undergoes reacting and ultimately resulting in $FCR^+$.

40µl of 0.6mM glucose solution was applied to individual sensors, with a potential of -0.2V applied after varying delay times.

Table 4.1Comparison of the incubation periods at open circuit.

| Delay Time (minutes) | Current (µA) |
|---|---|
| 1.5 | -9.326 |
| 3.0 | -10.032 |
| 5.0 | -9.283 |

Table 4. 1 shows that increasing the incubation time above 3 minutes, did not have an impact on the results and therefore the 3 minutes incubation time was optimal.

4.2.4.5 Comparison of the Glucose Biosensor to the commercially available analyser (YSI-Analyser).

At present the industry uses the YSI, as the industry “golden standard”. It was decided that this was the best was the best method to method to compare the biosensor with. The biosensors where prepared and calibrated (using triplicate readings on multiple analytical glucose concentrations). The concentration obtained by the biosensor was calculate by the manipulation of the current by the calibration gradient of the biosensor.

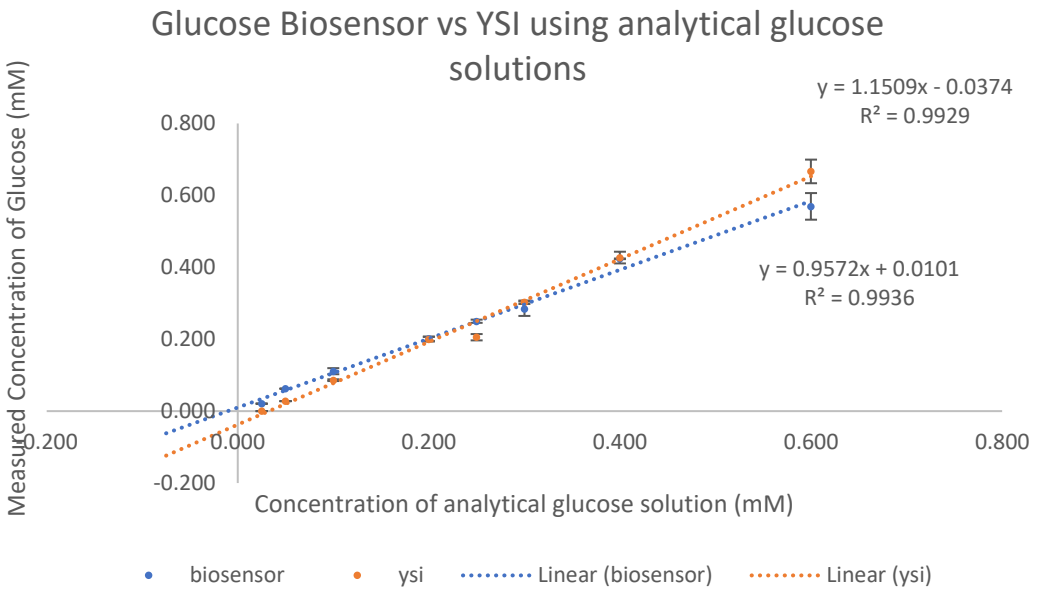



*Figure 4.14 Comparison of Glucose Biosensor and YSI using analytical solutions.*

Using analytical solutions of glucose, the glucose biosensor was compared with the current market leader product.

YSI $\left|\frac{1-1.1509}{1} \times 100\right|$ = 15.09 % correlation deviation

Proposed Biosensor $\left|\frac{1-0.9572}{1} \times 100\right|$ = 4.28 % correlation deviation

The proposed glucose biosensor gives a much closer value to the standard glucose solutions than the competitor product (YSI Analyser).

Another important point is that the YSI intercepts the x-axis at 0.032 mM, therefore any value below this would produce a reading of zero. However, the biosensor intercepts much closer to zero at -0.011 mM. It is crucial to recognise that the YSI

provided a value of 0.000 mM, for all tests of 0.025 mM of glucose analytical solution. This is due to the limit of detection, of the YSI system, being around 0.100 mM.

Table 4.2 Comparison of Glucose Biosensor and YSI using low sugar variety potatoes (VR808 variety, a low sugar producing potato).

| Repeat Number | Glucose Concentration Measured (mM) | |
|---|---|---|
| | Biosensor | YSI |
| 1 | 0.094 | 0.121 |
| 2 | 0.094 | 0.127 |
| 3 | 0.106 | 0.127 |
| 4 | 0.111 | 0.173 |
| 5 | 0.100 | 0.172 |
| 6 | 0.094 | 0.162 |
| 7 | 0.100 | 0.117 |
| Average | 0.100 | 0.143 |
| St. Dev | 0.006 | 0.025 |
| CV% | 6.415 | 17.543 |

From the Table 4.2, it can be seen that the glucose biosensor gives more reproduceable results, than the commercially available system that is recognised as the “industry gold standard”. The glucose provided a co-efficient of less than 7% whilst the YSI was over 17% in comparison.

### 4.2.5 Analytical Application – Sucrose Biosensor

#### 4.2.5.1 Conversion of a Glucose Biosensor to a Sucrose Biosensor

The sucrose biosensors employ the same mechanism as the glucose biosensors with an additional step; this step involves invertase (an enzyme that converts sucrose to α-D-glucose and β-D-fructose).

As the system measures the glucose produced from the conversion of sucrose (by invertase) and the glucose already present, in the test solution, the sucrose biosensor cannot be measured in isolation from the glucose biosensor. The concentration of glucose (measured by the glucose sensor) is required to be subtracted from the total concentration produced by the sucrose sensor, to give the concentration of glucose generated from the sucrose molecules, hence the concentration of sucrose.

In plants, starch is synthesised from molecules of α-D-glucose, whilst cellulose is synthesised from β-D-glucose.

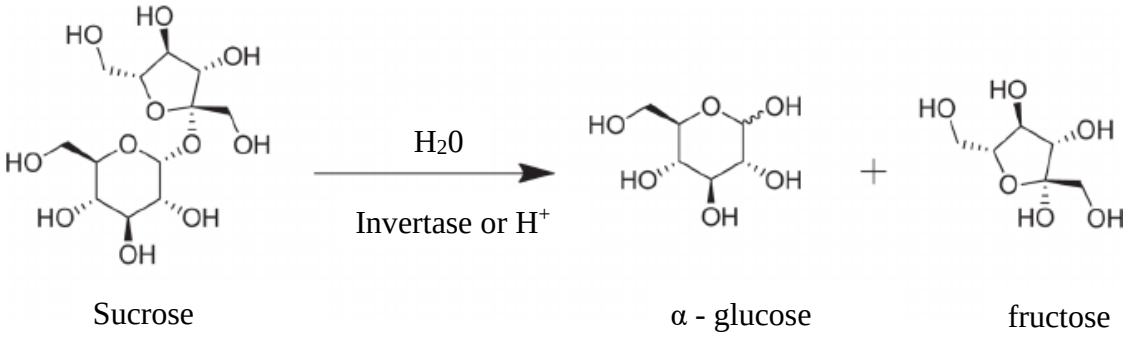



*Figure 4.17 Enzymatic Reaction of Sucrose and Invertase*

The form found in sucrose is α-D-glucose, and produces this form when cleaved, by enzymes such as invertase.

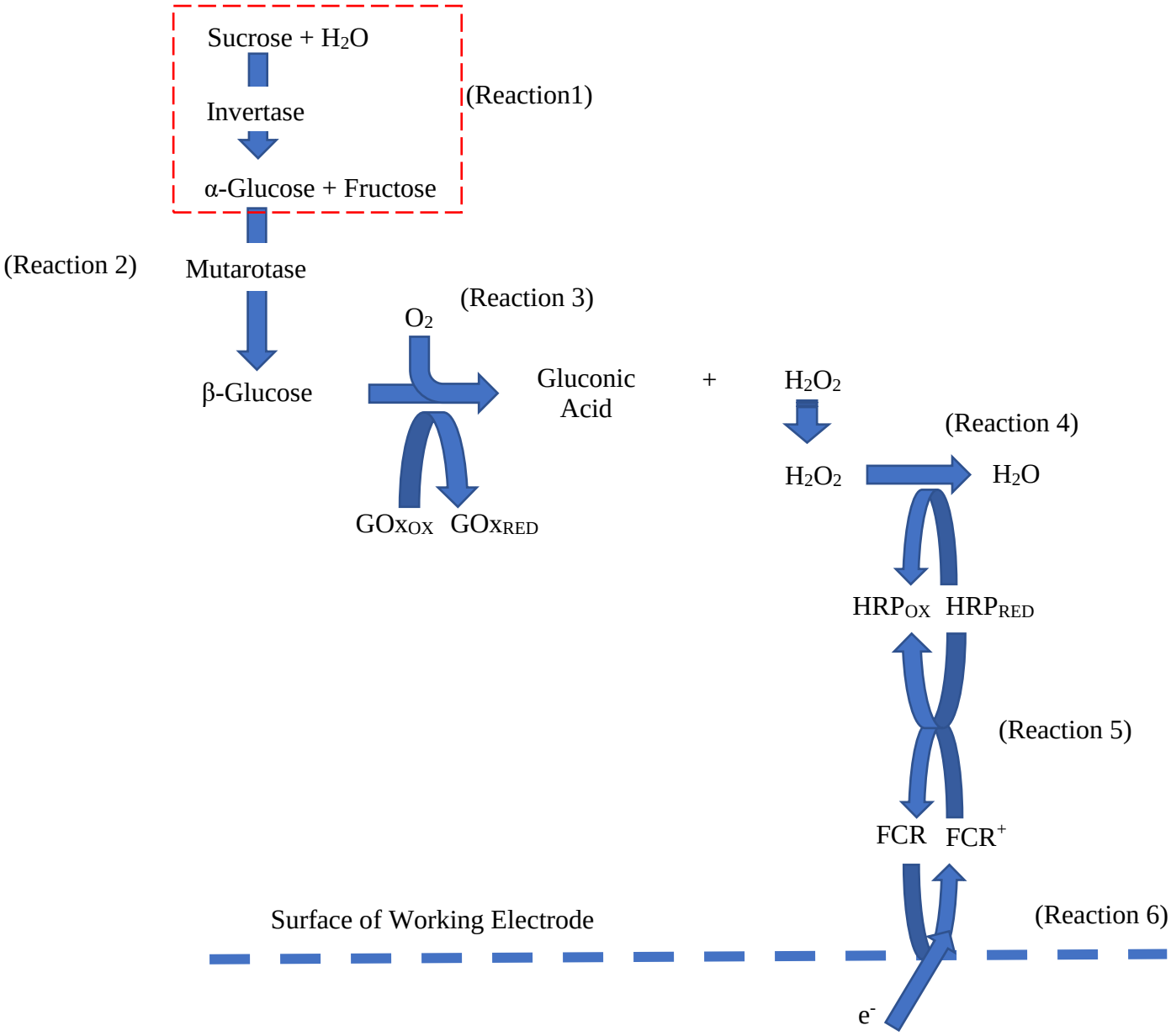



*Figure 4.18 The conversion of the glucose biosensor to a sucrose biosensor, by the addition of the invertase enzyme*

1) Sucrose + $H_2O$ + Invertase → α-Glucose + Fructose + Invertase
2) α-Glucose + Mutarotase ⇋ β-Glucose + Mutarotase
3) β-Glucose + $O_2$ + Glucose $Oxidase_{(Ox)}$ → Gluconic Acid + $H_2O_2$ + Glucose $Oxidase_{(Red)}$

4) $H_2O_2$ + Horseradish Peroxidase$_{(Red)}$ → $H_2O$ + Horseradish Peroxidase$_{(Ox)}$

5) Horseradish Peroxidase$_{(Ox)}$ + FCR → Horseradish Peroxidase$_{(Red)}$ + $FCR^+$

6) $FCR^+ + e^- \rightarrow FCR$

As the system uses the same mechanism, as the glucose sensor, there is a direct relationship between concentration of sucrose and the current produced. A sucrose calibration curve was carried out to ensure that the addition of invertase is effective.

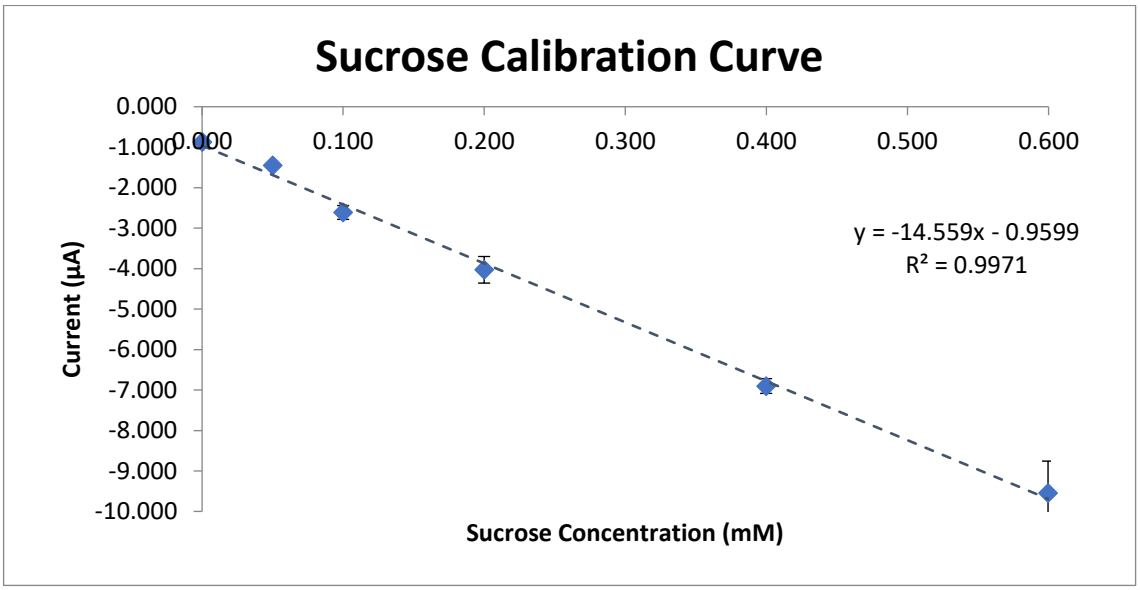



*Figure 4.19 Calibration curve obtained by the sucrose biosensor*

When sucrose is present in a test solution, the sensor will not only measure the concentration of sucrose but also the concentration of native glucose (glucose not generated by the invertase, from the sucrose molecules). To combat this, the glucose is required to be measured at the same time as sucrose. This way, the current produced by the sucrose molecules can be calculated by subtracting the

current produced by the glucose sensor from the current produced by the sucrose sensor.

4.2.5.2 Comparison with Commercially Available Analyser

Table 4.3

| Repeat Number | Sucrose Concentration Measured (mM) | |
|---|---|---|
| | Biosensor | YSI |
| 1 | 1.442 | 1.553 |
| 2 | 1.466 | 1.539 |
| 3 | 1.463 | 1.521 |
| 4 | 1.463 | 1.497 |
| 5 | 1.439 | 1.504 |
| Average | 1.455 | 1.523 |
| St. Dev | 0.013 | 0.023 |
| CV% | 0.868 | 1.541 |

From the Table 4.3, it can be seen that the glucose biosensor gives more reproduceable results, than the commercially available system that is recognised as the “industry gold standard”. Therefore, the former is a suitable approach for measuring sucrose concentration, in food samples.

## 4.3 Conclusion

This chapter describes the development of a simple, low cost chronoamperometric biosensor for glucose and sucrose, based on the electrocatalytic reaction of a novel ferrocene derivative. This derivative does not observe the same behaviour as over ferrocene-based mediators, as there is a lack of a competitive reaction between the $FCR^{+}$, the glucose oxidase and the working electrode. This provides a system that can measure concentrations of glucose and sucrose lower than 0.1mM, were other systems are unable to. The lower concentration allows the ability to measure new low sugar crops, such as low sugar potatoes designed for the crisping industry.

Consequently, the proposed biosensor could be adapted for a wide range of low sugar food products (and higher with the inclusion of dilution). It should be mentioned that sample preparation only involves the dilution of the sample prior to analysis, which is performed only on 40 µl, of solution, applied directly onto the sensor surface. Therefore, it is readily feasible that this approach could be performed near to the point of production and used as a quality control method.

## 4.4 References

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# Chapter 5

# Development and Designing of a Potentiostat System Suitable for Industry Use

Contents – Chapter 5

## 5.1 Introduction

As the sensors are designed for “in field” use, the system, which measures and presents the data needs to be suitable for unskilled personnel. Therefore, a potentiostat system is required for use with the biosensors to be used outside the laboratory and which is easy to use.

At present the biosensor measurements have been carried out using a commercial potentiostat, AutoLab [μAutoLab Type II], connected to a computer. The software that produces data also requires further manipulation to display the information that is more appropriate to the industry. The software produces chronoampergrams, and the user would have to take the reading (current) at a particular time, the current obtained requires manipulation by adding the intercept value (from the sensor calibration data) and then dividing by the gradient value. This value represents the value in millimoles (mM), depending on the industry (e.g. the food industry) the value may have to be converted to another unit, such as milligrams per litre (mg / l) or as a percentage of the produce (%). This can readily be achieved with the appropriate manipulation of the data, with a suitable program.

Furthermore, the current produced is required to be within the linear range of the sensors. Clearly, a current outside the linear range of the sensor will give incorrect values (when converted to concentration [mM]). Consequently, understanding the

linear range and detection limit of the system is crucial for ensuring that reliable results will be achieved.

If the current is beyond the linear calibration range then the sample solution would have to be diluted, until the current is within the linear range. The concentration will then be required to be multiplied by the dilution factor.

Another possible issue at present is the requirement to subtract the interference signal from the final result, for the fructose sensor. This would require the user to subtract the current of the interference sensor from the current obtained with the fructose sensor.

In the case of the glucose and sucrose sensor system, the currents are first required to be converted to concentrations. Then, the glucose concentration is subtracted from the sucrose concentration to produce the true concentration of sucrose, in the sample. This is due to the sucrose sensor measuring both glucose and sucrose in the sample.

There are many examples of biosensors that are measured in portable devices. The most common portable devices are for blood-glucose measurements, used by diabetics. The utilisation of an Android tablet and software would allow the user to enter additional details, that would specific to the testing. Details such as additional dilutions introduced by the sample preparation procedure.

For these reasons a simplified analytical procedure was considered to be required for the agri-food industry and will be described in the following sections

## 5.2 Proposed System

The proposed system was initially comprised of a single unit, with an internal OEM (Original Equipment Manufacturer) potentiostat and a built-in display with a series of buttons as interfaces. On the side of the unit, there are two connector ports, designed for the sensors.

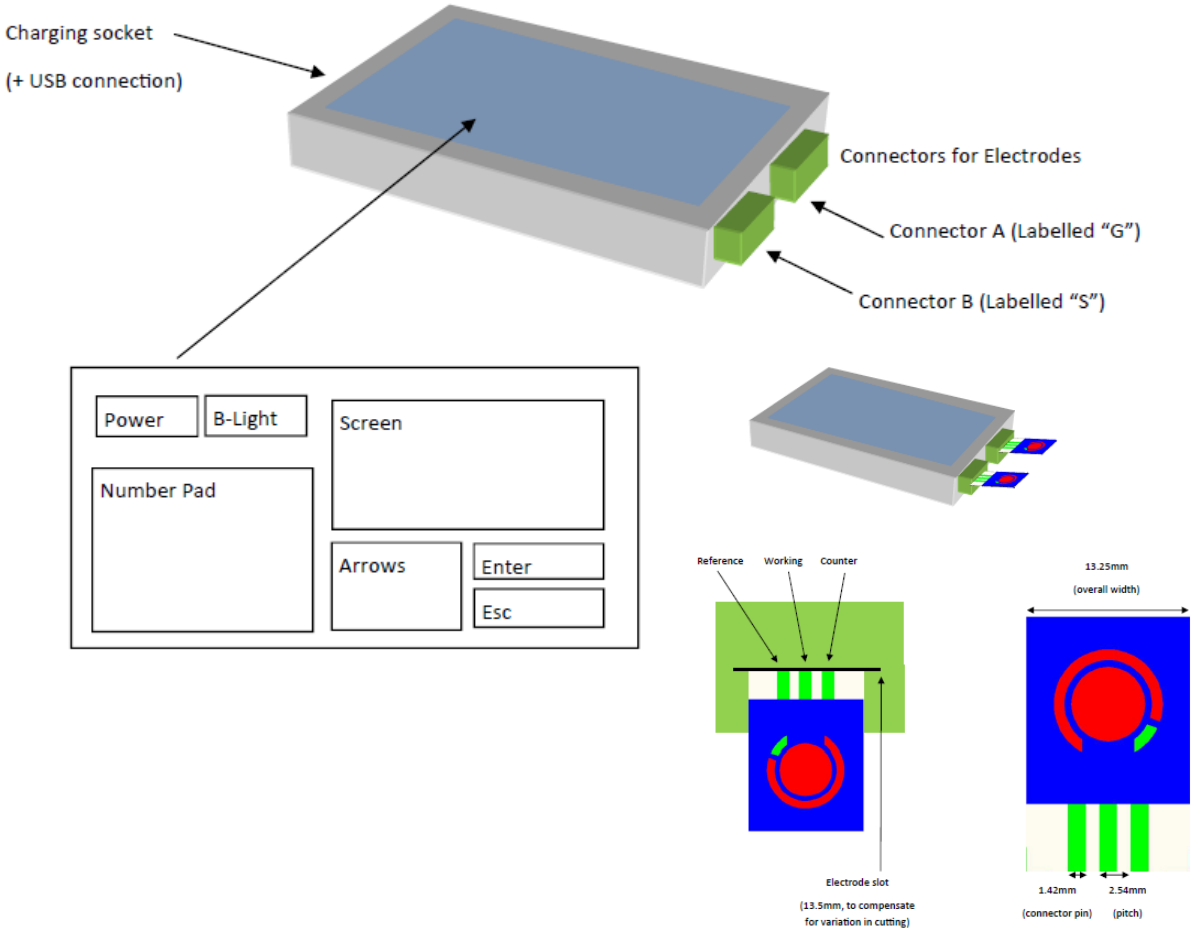


*Figure 5.1 Initial design concept of how the potentiostat system should function. See appendix 7.2 for high resolution and original design documentation.*

To reduce cost and to make the system easy to update, an OEM potentiostat with built in Bluetooth was purchased and modified accordingly. The modification included a rerouted PCB board, for the working, counter and reference connections to the D-sub port along with a signal connection for the micro-switch controller. A purpose-built connector head was designed and developed, containing two connector ports for two sensors to be placed in the device simultaneously. The connector head was modified to designed to have a micro-switch along with an electronic mechanism that triggers it. When the micro-switch is in position 0, the left hand side connector (labelled as “A” in the image) port is active and when the micro-switch is position 1, the right hand side connector (labelled as “B” in the image). This mechanism allows the first biosensor to be measured and then the micro-switch can then measure the second biosensor. This approach operates as a pseudo simultaneous (appears to be measuring at the same time to the end user, but actually measures one after the other). The advantage of this approach eliminates for two potentiostats/a multi-potentiostat thus making the system cheaper and more appealing to the consumer on a commercial level.


*Figure 5.2 A photograph of the finalised potentiostat system product*

As the use of android based tablets and mobile phones have increased in popularity, within the past decade [1-3], an application was designed and built to interact with the potentiostat and manipulate the data. Another advantage of using this system is that the end user can use a compatible mobile phone or tablet, making it more appealing and economically. The user can install the apk file (android package kit) onto any compatible android device.

The software was designed to be used on Android devices, the use of apk files allows the software to be easily be installed and tested onto the devices without the necessity of distributing it through the official online distribution stores. The software was written in pseudocode (see appendix 7.2 for pseudocode and original concept designs). Apple devices were considered but this would require the application to be distributed via the online store, making the trials and adjustments more complex.

During trials with non-technical users, one of the comments/issues was the inputting of the calibration data, which was unique to each batch of sensors. An example of the data that was to be entered by the user is below:

E.g. for a typical glucose biosensor, the label will contain the following information:

A: -26.191

B: -1.1825

C: -9.0066

D: -1.9375

Where A is the gradient (μA $mM^{-1}$), B is the intercept (μA), C is the upper limit of the linear range (μA) and D is the lower limit of the linear range (μA).

This information was provided so that the end user would not have to manually carryout a calibration study. The gradient and intercept value (unique to each batch of sensors) is required. In addition, the lower and upper limits of the linear range are required. There parameters are carried out and coded into the QR-code, before distribution to the user. Each batch of sensors contains these values on the label.

The main issue arises when the end user enters these values into the system, as there is a large possibility of transcribing errors of the calibration values, QR-code (Quick Read Code) would be included with each batch of sensors.


*Figure 5.3 Screenshot of the QR-code being read from the tablet camera*

To make the system more user friendly, the front camera of the android device was utilized for inputting the batch details. Within the android application the user will be asked to QR-code, for the calibration data (previously tested and programmed into the QR-code, before distribution to the customer and unique to each batch of sensors), batch number of the sensors and check that the correct type of sensor is being used. Once the batch details are scanned onto the device, they can be referred to by the user for subsequent measurements selected.

As the above system reads the current from two biosensors simultaneously in theory two potentiostats (within a single unit) would be required. However, to make the system more economically viable, a single potentiostat (within the unit) was used in this project, keeping costs down. A “pseudo-simultaneous” approach was used, which involves the sensors being applied in end point reactions and not

relying on readings taken during a kinetic reaction. If the sensors are interrogated for a few seconds longer than the allocated incubation time, any slight changes in this incubation time will not have any detrimental effect on the results. This is due to the reactions already being complete.

Therefore, one biosensor reading can be read and the followed by the second biosensor reading, as shown in Figure 5.4. This eliminates the need for the user to measure one sensor and then repeat the process for the second biosensor. Thus, appearing to be simultaneous to the end user.

Below is a schematic for the timing system for glucose and sucrose measurement.

**Time schedule during Testing (Testing Samples Menu System screen6)**

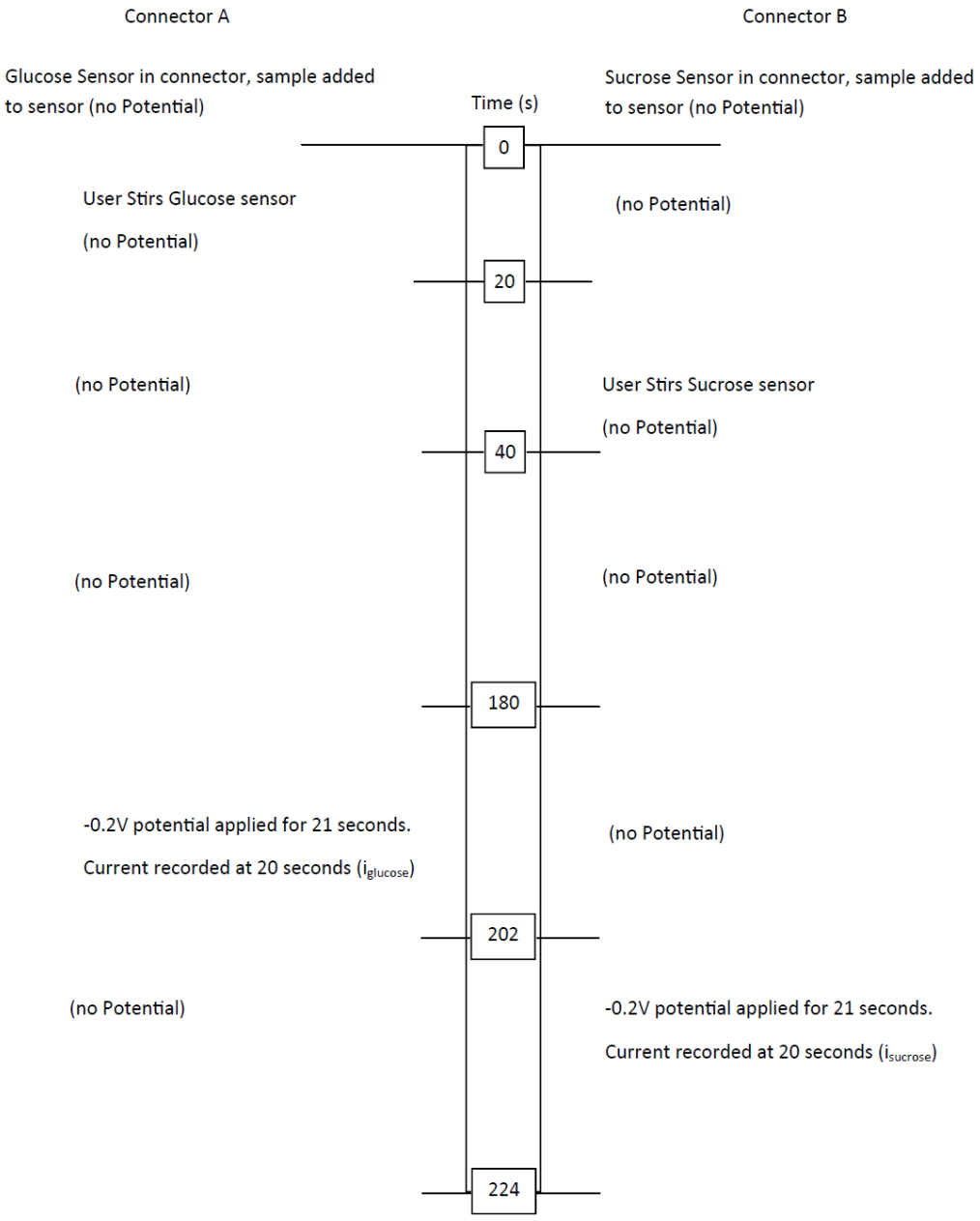


*Figure 5.4 Schematic for the timing system for the glucose and sucrose measurement.*

It should be emphasized that another advantage of using an android application is that the data can be saved to the phone or tablet and presented as an excel spreadsheet.

## 5.3 Experimental

### 5.3.1 Chemical reagents

Mutoratose, glucose oxidase and horseradish peroxidase were obtained from BBI Solutions. (www.bbisolutions.com)

The ferrocene based mediator was obtained from Gwent Electronic Materials. (www.gwent.org).

All other chemicals and reagents were obtained from Sigma-Aldrich (UK). (*www.sigmaaldrich.com)*

McIlvaine buffer was prepared by mixing 0.2 M citric acid (containing 0.2 M KCl) with 0.4 M disodium phosphate (containing 0.2 M KCl) to produce a final pH of 4.5.

D-fructose dehydrogenase was obtained from Toyobo Enzymes (Japan). (www.toyobo-global.com)

The graphite-nanoparticles (graphite modified with carboxylic acid) in solution (C2131210D1) were obtained from Gwent Electronic Materials. (www.gwent.org).

All other chemicals and reagents were obtained from Sigma-Aldrich (UK). (*www.sigmaaldrich.com)*

### 5.3.2 Apparatus and Instrumentation

All electrochemical measurements were conducted with a two-electrode system, consisting of a screen-printed working electrode (GEM code: C2030519P4), Ag/AgCl reference electrode (GEM Product Code: C61003P7) both screen-printed onto valox (a semi-crystalline material based on polybutylene terephthalate and polyethylene terephthalate polymers; Cadillac Plastics Swindon, UK). The diameter (6 mm) of the working electrode was defined using a dielectric ink (GEM Product Code: D2070423P5) a concentric silver/silver-chloride served as the counter/reference electrode, (GEM Electrode Design: BE2110916D1).

*OEM Potentiostat details are unavailable due to industry trading agreements*

## 5.4 Results and Discussion

To ensure that the new potentiostat system is suitable to use and correctly functioning, the system was compared with results obtained from the autolab potentiostat.

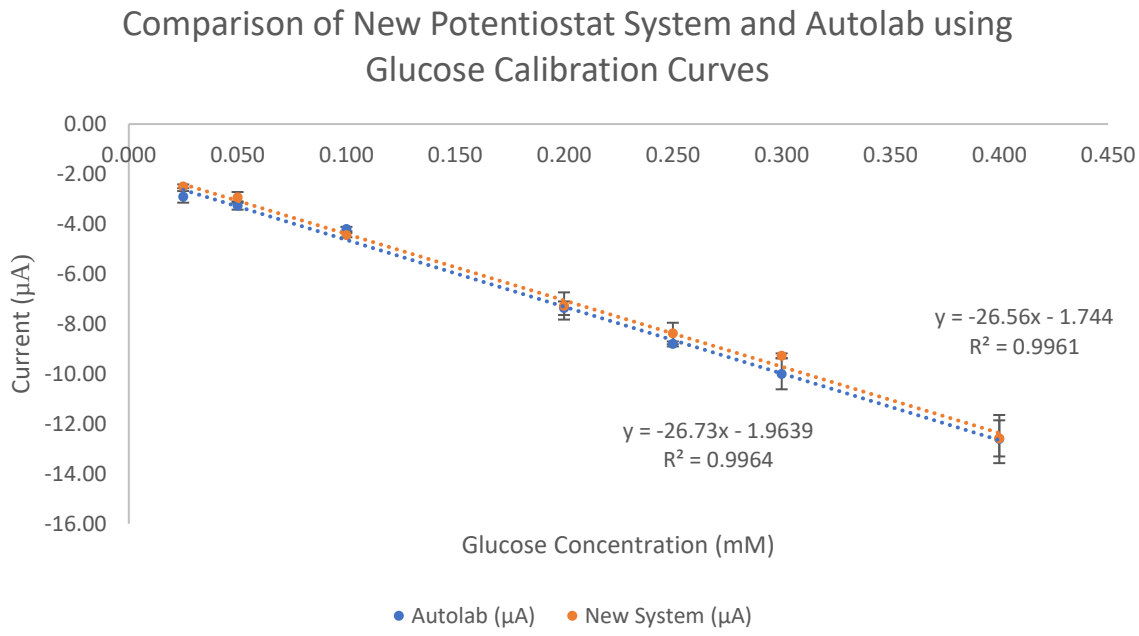


*Figure 5.5 Comparison of Glucose Calibration Results*

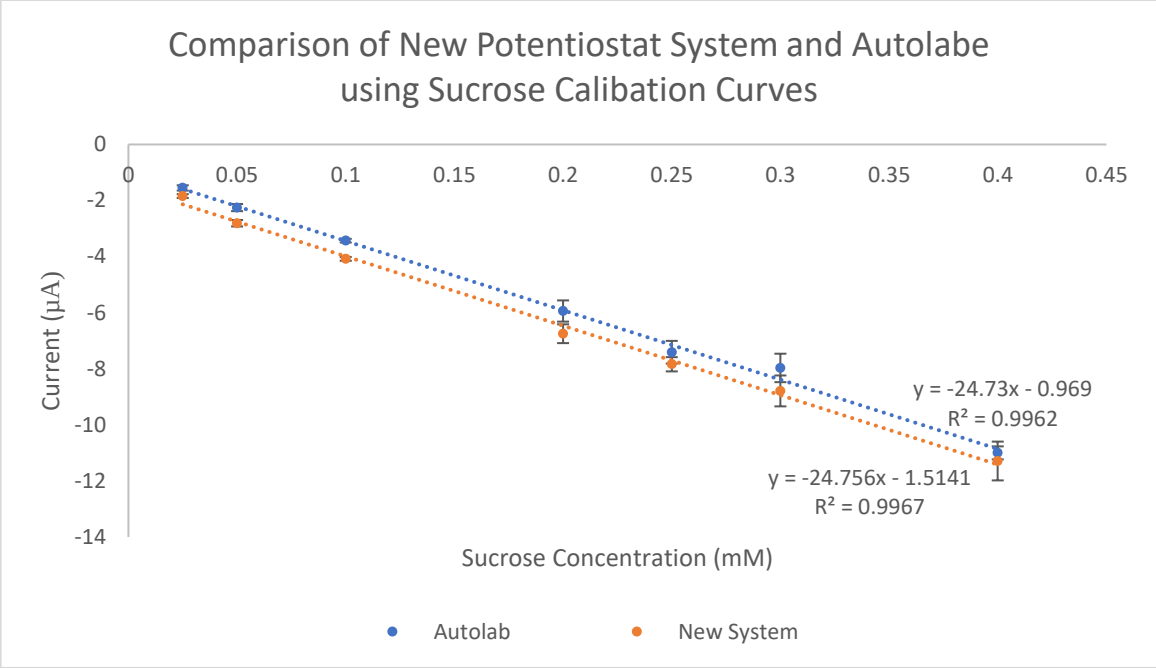


*Figure 5.6 Comparison of Sucrose Calibration Results*

For the fructose application, the requirements are slightly different. This is due to several factors such as the system working in the oxidative state instead of the reductive state. Therefore, the currents are in positive values instead of negative values. The values for the upper limit and lower current values are also positive numbers, for the readings to be in range.

Another difference is that instead of the “sucrose” sensor subtracting the “glucose” sensor, Fructose A (biosensor with fructose dehydrogenase) will subtract Fructose B (background/interference signal measurement).

To ensure that the user is using fructose A sensors and fructose B sensors from the same batch, the application will check to see whether the same batch number

is scanned for the two different sensors/pots/QR-codes. If they are not the same batch number, then the application will not allow the user to continue with the process.

The reason for the same batch to be used to ensure that the same buffer concentration and ferricyanide solution is deposited, to ensure that there is no batch to batch variation.

As with the glucose and sucrose sensors, the fructose sensors are calibrated and tested before distribution to the end user.

## 5.5 Conclusion

This chapter describes the development of a handheld potentiostat system, with a dedicated android application installed on an android tablet.

The proposed biosensor system has been found to be suitable for food products, such as industrial potatoes for crisp manufacturing. Therefore, it is readily feasible that this approach could be performed near to the point of production and used as a quality control method and a storage monitoring system.

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# Chapter 6

# Conclusion & Future Work

Contents – Chapter 6

## 6.1 Conclusion

This thesis describes the construction of biosensors, for the sugars: glucose; fructose; and sucrose. This thesis also comprises the construction of a system that can be used by the industry, to measure these sugars in food produce that contain them in low concentrations (of less than 1 mM).

Chapter 2 describes the development of a simple, low cost chronoamperometric assay, based on the electrocatalytic oxidation of FDH with a SPCE-G-COOH, and evaluation using a commercial fruit juice. It describes how the introduction modified graphite nanoparticles was found to enhance the sensitivity and improved the precision of the response towards the enzymatically generated ferrocyanide species, compared with a plain SPCE. This providing the advantage of improved sensitivity, resulting in better resolution between different concentrations.

The fructose calibration studies indicated that a linear response could be obtained up to 1.00 mM fructose using a mediator concentration of 3 mM. Consequently, the proposed biosensor could be adapted for a wide range of food products. It should be mentioned that sample preparation only involves the dilution of the sample prior to analysis; which is performed only on 40 µl, of solution, applied directly onto the sensor surface. Therefore, it is readily feasible that this approach could be performed near to the point of production and used as a quality control method.

Chapter 2 provided an approach to the measurement and detection of fructose concentrations, using a bioassay. Although this method worked, it using multiple solutions making it less practical for use in the field or by non-skilled personnel.

Chapter 3 combats these issues by describing the conversion of the fructose chronoamperometric assay to a biosensor system. This was achieved by the drying down of the fructose dehydrogenase and the mediator, onto the surface of the working electrode. The end user then only needs to apply 40 µl of the test sample and reconstitute the enzyme and mediator into the solution via agitation.

One potential issue was when the test sample had elevated concentrations of ascorbic acid. As this molecule could be detected at the same potential as the mediator, this interferent could affect the results. As a result, the system was adapted to analyse samples containing higher ascorbic acids concentration closer to the concentration of fructose. The approach found to eliminate this issue was the use of a linear subtraction two sensor/biosensor system. This subtracts any electrochemical signal produced from interference molecules, such as ascorbic acid.

Furthermore, Chapter 3 also increased the sensitivity of the system (from 15.525 µA/mM to 26.818 µA/mM by using the chronoamperometric current measured at 5 seconds, instead of 20 seconds.

The proposed biosensor system has been found to be suitable for food products, such as industrial potatoes for crisp manufacturing. Therefore, it is readily feasible that this approach could be performed near to the point of production and used as a quality control method.

Chapter 4 describes the development of a simple, low cost chronoamperometric biosensor for glucose and sucrose, based on the electrocatalytic of a novel ferrocene derivative. This approach utilises the measurement of the mediator in the reduction phase of -0.2 V. At this potential the likelihood of interferences in low, this was achieved in this system but not in the fructose biosensor is due to the use of hydrogen peroxidase. The fructose biosensor uses a dehydrogenase enzyme and thus not interact with the ferrocene-based mediator. Consequently, the proposed biosensor could be adapted for a wide range of food products. It should be mentioned that sample preparation only involves the dilution of the sample prior to analysis; which is performed only on 40 µl, of solution, applied directly onto the sensor surface. Therefore, it is readily feasible that this approach could be performed near to the point of production and used as a quality control method.

Chapter 5 describes the development of a handheld portable system, comprised of a modified potentiostat with a dedicated android application installed on an android tablet.

The proposed biosensor system has been found to be suitable for food products, such as industrial potatoes for crisp manufacturing. The system allows the user to measure either glucose and sucrose together or fructose on its own. Therefore, it is readily feasible that this approach could be performed near to the point of production and used as a quality control method and a storage monitoring system.

Overall, the system is suitable as a direct competition that is already present on the market, producing a cheap and easy alternative for the end user for glucose and sucrose. In terms of measuring fructose, it is a new entry to the market as a portable system to measure fructose concentrations.

*The system has been independently validated and is being used by a large company, in the food industry. Due to industry non-discloser agreements, the values and reports are not allowed to be disclosed in this thesis.*

## 6.2 Future Work

### 6.2.1 Eliminating Human Error from the Stirring Phase

At present all the sensors require agitation via the user mixing the test sample and biosensor components with a mixing rod. This can provide issues such as the amount of pressure applied by the end user, the rate of moments and contamination (from mixing one sensor to another).

In order to make the components to mix more efficiently could be the incorporation of a non-ionic surfactant to the sensor. Several studies would be required to be carried out, to ensure that no interference signal is introduces and to optimise the loading of the surfactant for a significant effect. This method has been used in biosensors, to make the agitation easier [1].

Another idea is the addition of a vibrating disc motor, to the potentiostat unit. The vibrating disc motor would be located under the sensor and controlled by the tablet application. This would provide a consistent agitation between tests and the time of agitation would be more closely controlled. A vibration module to potentiostats have been used as a technique, for a “self-agitation” system, in current systems [2]. A combination of surfactants and a vibration motor will result in more consistent results and eliminating some of the human error.

### 6.2.2 Remove the Requirement for a Micro-pipette

The current system for all the biosensors requires the use of a micro-pipette, to dispense 40 µl of the test sample on to the biosensor. For “field-use” and for low-skilled users, the use of the micro-pipette is a possible source of issues and also the introduction of errors and variability. An attractive solution is to apply a capillary fill system, using surface tension of the test solution to fill a set volume, on the biosensor. This has been implemented into numerous biosensors, including sensors for blood-glucose, glutamate and other biosensors [3-6]. This will require a lot of optimisation due to the current volume of test solution requiring 40 µl.

### 6.2.3 Decreasing the Incubation Time and Overall Running Time of the Biosensors

Currently, 3 minutes of incubation time may be “unattractive” as an industrial tool, due to the amount of time that the has to wait between applying the test solution and obtaining the result.

The incubation time could possibly be reduced to a more “attractive” incubation period of around 1 minute. This could be achieved by increasing the enzyme loading (increasing the number of units of each enzyme on the biosensors). This will cause the enzyme kinetics to work at a faster rate, reaching an end point reaction in a faster time.

The only issue with this maybe the cost of increasing the number of units of enzymes. If the manufacturing cost is too high, this could result in the product would not be cost effective for the end user. A balance between reducing the incubation time verses cost analysis would have to be investigated, in a commercial and industrial point of view.

### 6.2.4 The Glucose and Sucrose Biosensors Limited by Oxygen Concentration

For the glucose and sucrose biosensors, as mentioned in Chapter Four, is that oxygen is a limiting factor for both systems. This restricts the linear range of the concentrations that can be measured.

Ideally subjecting the test samples with oxygen gas, before testing, would solve this issue. However, this approach is not suitable as a commercial product due to the not being a practical method for “field-use”, along with the health and safety aspect.

The addition of an agent that produced oxygen, on the biosensor, is a possible solution. A series of tests on a suitable agent would have to be investigated and optimised.

### 6.2.5 Combining All the Biosensors into One “Chip”

Combing all the biosensors onto one “chip” is an attractive approach. However, this would only allow the end user to measure all three of the sugars, instead of selecting individual ones.

The produce an “all in one chip”, the system would require to a capillary fill system (as described in 6.2.2). The capillary fill system would have to split the system into individual channels (to the individual biosensors).

This approach will cause issues as the fluid would cause “cross-talk” between the different biosensors. The combat this effect, once the fluid have reached the individual biosensors, and no more fluid can be taken up by the capillary fill action, then the fluid would need to have a barrier between the biosensors.

The use of a hydrophobic printed layer could provide this barrier. Once the capillary action finishes “pulling” the fluid across the hydrophobic path, then the hydrophobic print would “split” the fluid and form a barrier of air. The air would have to come from a small vial directly above the hydrophobic path.

This would be arguably the most difficult enhancement to add. A lot of optimisation and testing would be required to see whether this is a suitable approach. The hydrophobicity of the path would have to also be investigate, so that is allows the fluid to be “pulled” over this path by the capillary effect but strong enough to separate the fluid afterwards.

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# Chapter 7

# Appendices

Contents – Chapter 7

## 7.1 Paper Submitted Based of Chapter 2

Development of a simple, low cost chronoamperometric assay for fructose based on a commercial graphite-nanoparticle modified screen-printed carbon electrode

Food Chemistry 241 (2018) 122–126

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# Development of a simple, low cost chronoamperometric assay for fructose based on a commercial graphite-nanoparticle modified screen-printed carbon electrode

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ABSTRACT

This paper describes the development of a simple, low cost chronoamperometric assay, for the measurement of fructose, using a graphite-nanoparticle modified screen-printed electrode (SPCE-G-COOH). Cyclic voltammetry showed that the response of the SPCE-G-COOH enhanced the sensitivity and precision, towards the enzymatically generated ferrocyanide species, over a plain SPCE; therefore the former was employed in subsequent studies.

Calibration studies were carried out using chronoamperometry with a 40 µl mixture containing fructose, mediator and FDH, deposited onto the SPCE-G-COOH. The response was linear from 0.1 mM to 1.0 mM. A commercial fruit juice sample was analysed using the developed assay and the fructose concentration was calculated to be 477 mM with a precision of 3.03% (n = 5). Following fortification (477 mM fructose) the mean recovery was found to be 97.12% with a coefficient of variation of 6.42% (n = 5); consequently, the method holds promise for the analysis of commercial fruit juices.

## 1. Introduction

The ability to precisely and accurately measure the sugar known as fructose has become of considerable interest, to many food companies. For example, the wine manufacturing industry use the concentration of fructose (along with glucose) to predict the alcohol content following fermentation (Bauer & Pretorious, 2000; Guillaume, Delobel, Sablayrolles, & Blondin, 2007). The fructose concentration in commercial fruit juices is also an important indicator of the freshness of the food product (Fadel, 2008).

Currently, few reports describe the development of amperometric assays for the measurement of fructose, compared with other sugars such as glucose and sucrose (Antiochia and Gorton, 2014; Biscay et al., 2012; Tsujimura, Nishina, Kamitaka, & Kano, 2009). One of the current methods, of determining fructose and other simple sugars, involves the °Brix test (Cejpek, 2012; Kawahigashi, Kasuga, Okuizumi, & Hiradate, 2013), which is based on refractometry; this provides the percentage of total dissolved solids present in the liquid sample. As this method involves refractive index measurements, alcohol can have a detrimental effect on the result, owing to the difference in refractive index between alcohol and water (Dongarea, Buchadeb, & Shaligramca, 2015). An alternative approach is based on Fourier transform infrared spectroscopy (Reru, Wibowo, & Rondonuwu, 2016; Wang, Kliks, Jun, Jackson, & Li, 2010), however this technique is not readily applicable to remote analysis and has a relatively high cost.

An attractive alternative approach, which we decided to explore, involves the development of a simple chronoamperometric assay, based on a screen-printed electrode. This is a low cost method, particularly when carbon materials are used in the fabrication of the electrodes. Screen-printed carbon based sensors have been previously developed by our group for the measurement of a wide variety of analytes, (Hughes, Pemberton, Fielden and Hart, 2016; Hughes et al., 2016). We recently demonstrated the possibility of measuring the sugar galactose, using the enzyme galactose oxidase in conjunction with a screen-printed carbon electrode, modified with the mediator cobalt phthalocyanine (Kanyong, Hughes, Pemberton, Jackson, & Hart, 2016; Kanyong, Pemberton, Jackson, & Hart, 2013). In another paper, we demonstrated the possibility of developing a biosensor for the measurement of glutamate, in a food sample using the enzyme glutamate dehydrogenase integrated with a screen printed carbon electrode. It was possible to carry out the analysis of commercial OXO cubes, after a very simple dissolution and dilution step (Hughes, Pemberton, Fielden, & Hart, 2015).

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0308-8146/ 

Consequently, we decided to explore the possibility of developing a simple electrochemical sensor system, for the measurement of fructose in food samples, based on screen-printed carbon electrodes (SPCEs) in conjunction with fructose dehydrogenase. As the incorporation of nanoparticles in the chronoamperometric measurement of glutamate proved to be advantageous, we decided to investigate a novel nanomaterial in the present study.

This paper describes the optimization of the components and operating conditions, of a chronoamperometric assay for fructose; this incorporated fructose dehydrogenase with a nanoparticle modified screen-printed electrode. The possibility of measuring the sugar, in a commercial fruit juice, will be discussed.

## 2. Experimental

### 2.1. Chemical reagents

D-Fructose dehydrogenase was obtained from Toyobo Enzymes (Japan). (www.toyobo-global.com).

The graphite-nanoparticles (graphite modified with carboxylic acid) in solution (C2131210D1) were obtained from Gwent Electronic Materials. (www.gwent.org).

Apple juice was obtained from a local supermarket.

All other chemicals and reagents were obtained from Sigma-Aldrich (UK). (www.sigmaaldrich.com).

McIlvaine buffer was prepared by mixing 0.2 M citric acid (containing 0.2 M KCl) with 0.4 M disodium phosphate (containing 0.2 M KCl) to produce a final pH of 4.5.

### 2.2. Apparatus and instrumentation

All electrochemical measurements were conducted with a two-electrode system, consisting of a screen-printed working electrode (*GEM* code: C2030519P4), Ag/AgCl reference electrode (*GEM* Product Code: C61003P7) both screen-printed onto valox (a semi-crystalline material based on polybutylene terephthalate and polyethylene terephthalate polymers; Cadillac Plastics Swindon, UK). The diameter (6 mm) of the working electrode was defined using a dielectric ink (*GEM* Product Code: D2070423P5) a concentric silver/silver-chloride served as the counter/reference electrode, (*GEM* Electrode Design: BE2110916D1). For further studies, the surface of the working electrode was modified by addition of 10 µl of graphite-nanoparticles (1.787 mg $ml^{-1}$) (*GEM* code: C2131210D1).

The working and reference electrodes were connected to the potentiostat with *GEM* electrode connector (*GEM* Code: CON002). All electrochemical studies were performed using an AutoLab [µAutoLab Type II], with General-Purpose Electrochemical Software (The Netherlands). Data were further analyzed with Microsoft Excel.

Fig. 1 summarises the fabrication and operation of the fructose biosensor.

It should be noted that during the fabrication of the nano-particle modified electrodes, the deposited nano-particles were confined to the working area by the hydrophilicity of the carbon and the hydrophobicity of the underlying valox substrate. This ensured that the working area remained the same between the unmodified and modified working electrodes and was confirmed by visual inspections.

### 2.3. Procedures

Cyclic voltammetry was performed by depositing a 300 µl aliquot of 0.5 mM ferricyanide, in 0.1 M phosphate buffer pH 7.5 containing 0.1 M potassium chloride onto the surface of the screen-printed carbon electrodes. Cyclic voltammetry was performed using the following conditions: initial and final potential +0.8 V; switching potential −0.4 V; scan rate 10 mV $s^{-1}$. Potential held at +0.8 V for 20 s before initial cycle.

Calibration studies were performed using chronoamperometry with standard solutions of fructose, over the concentration range 0.20–32.00 mM, in water; FDH was dissolved in McIlvaine buffer to produce concentrations of either 50 U $ml^{-1}$ or 200 U $ml^{-1}$. The measurement procedure involved the deposition of 20 µl of either enzyme solution, onto the screen printed transducer, followed by 10 µl of 12 mM ferricyanide and 10 µl fructose standard. Following an incubation time of 180 s (open circuit), with initial 20 s of agitation, the potential was stepped from open circuit to +0.3 V vs Ag/AgCl. Currents were measured 20 s after application of the voltage and these values were used to plot calibration graphs.

### 2.4. Analytical application

A preliminary study was performed with the commercial apple juice, to deduce an appropriate dilution procedure. A series of dilute apple juice solutions were prepared by mixing the neat sample with deionized water to produce final dilutions in the range of 1/2 and 1/512, of the original concentration. The analysis was carried out using chronoamperometry as described above, and from the results the optimum dilution that produced a signal within the linear range was deduced.

The method of standard addition was performed with the optimum dilution of the apple juice (with deionized water). This was achieved by mixing the diluted apple juice with different concentration fructose standards, so that the final concentration of the standard added was between 0.1 and 0.8 mM. This procedure was repeated 5 times, with each data point being replicated 5 times. The concentration of fructose in the original sample was obtained from this data, together with the precision of the measurements.

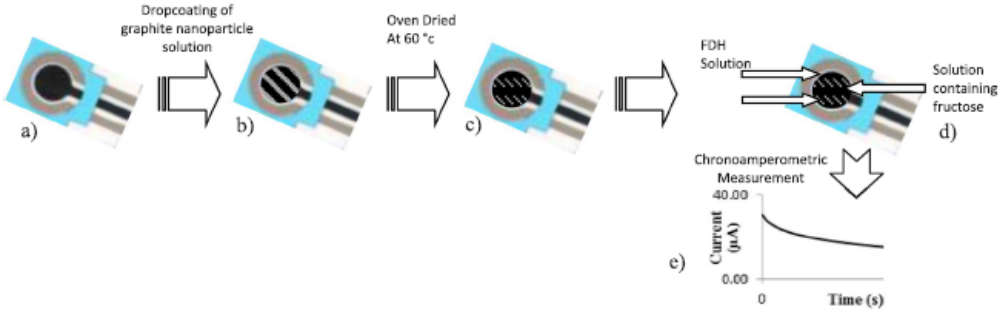


**Fig. 1.** Scheme showing the fabrication of the fructose biosensor and chronoamperometric measurement of fructose: a) Plain SPCE; b) SPCE with deposition of nanoparticles in solution; c) SPCE with dried nanoparticles; d) addition of 10 µl FDH, 10 µl ferricyanide; 20 µl of solution containing fructose; e) Chronoamperometric measurement.

The recovery of added fructose was ascertained by spiking the original sample with 477.2 mM of fructose (this was equal to the concentration found in the undiluted sample). The same chronoamperometric procedure was used, as previously described.

## 3. Results and discussion

### 3.1. Principles of the amperometric measurement of fructose

$$\text{Fructose} + FDH_{Ox} \rightarrow FDH_{Red} + \text{5-dehydro-D-fructose} \quad (1)$$

$$FDH_{Red} + 2Fe^{3+} \rightarrow 2Fe^{2+} + FDH_{Ox} \quad (2)$$

$$2Fe^{2+} \rightarrow 2Fe^{3+} + 2e^{-} \quad (3)$$

Eqs. (1–3) summarise the sequence of reactions involved in the chronoamperometric measurement of fructose, at the surface of a SPCE-G-COOH. Initially, D-fructose dehydrogenase ($FDH_{Ox}$) is reduced by fructose (Eq. (1)) and this reduced form of the enzyme ($FDH_{Red}$) reduces ferricyanide ($Fe^{3+}$) to ferrocyanide ($Fe^{2+}$) (Eq. (2)); the oxidized form of the enzyme ($FDH_{Ox}$) is regenerated during the latter reaction. At a potential of +300 mV, ferrocyanide is oxidised back to ferricyanide (Eq. (3)), resulting in the analytical response. Prior to the application of the applied potential, an incubation time of 3 min is allowed for the enzymatic oxidation of fructose; the reaction involving ferricyanide results in the conversion of two molecules of the mediator for every molecule of fructose. The magnitude of the resulting electrocatalytic current is proportional to the concentration of fructose, over the range of interest.

### 3.2. Cyclic voltammetric behaviour

An initial study was performed in order to investigate the effect of modifying the SPCE surface with COOH-graphite nanoparticles. The nanoparticles were drop coated onto the plain screen printed electrode (and dried) and interrogated by cyclic voltammetry, using a solution of 0.5mM ferricyanide, in 0.1 M phosphate buffer pH 7.5 containing 0.1 M potassium chloride.

Fig. 2 shows a comparison of the cyclic voltammetric behaviour of an unmodified screen printed carbon electrode (a) and a SPCE with 17.89 μg deposited onto the surface (b). The increase in current magnitude may be explained by an enhancement in the electron transfer properties, from ferrocyanide to the modified electrode surface. It should be mentioned that the coefficient of variation was determined for the anodic peak currents; this was found to be reduced from 6.73% to 4.75% (n = 9), for plain SPCE and SPCE-G-COOH, respectively. For the development of the chronoamperometric assay this improved precision, together with the improved sensitivity, can be considered of importance to the development of a reliable analytical procedure.

Fig. 2 also shows that there is a difference in the peak separation ($\Delta E_p$) for the two voltammetric scans, for the plain SPCE and SPCE-G-COOH; these values were 169 mV and 148 mV, respectively. Both values indicate that the redox reaction is quasi-reversible; however, the reaction at the SPCE-G-COOH appears to be more favourable. Further evidence for this is obtained the charged transfer coefficient (α), using Eq. (4) (Kirsch, Hart, Bird, Luxton, & McCalley, 2001) where is n is the number of electrons involved in the rate determining step.

$$\alpha n = \frac{0.048}{E_p(V) - E_{\frac{p}{2}}(V)} \quad (4)$$

The α values (obtained using n = 1) for the plain SPCE and SPCE-G-COOH were calculated to be 0.56 and 0.64, respectively. Therefore the electron transfer kinetics are more favourable in the case of the SPCE-G-COOH, which indicates that this sensor has superior electrochemical characteristics, which should lead to more reproducible measurements. It should be mentioned that both of these values are better that reported for a commercial graphite electrode (reported α = 0.486) (Botasini, Marti, & Méndez, 2016). From these results, it appears that the reason for the increase in current magnitude occurs as a result of improved electron transfer, rather than a simple increase in electrode surface. Consequently, all further studies were performed using the SPCE-G-COOH electrodes.

### 3.3. Calibration studies, using chronoamperometry with a SPCE-G-COOH

We began this study by investigating the SPCE-G-COOH modified with 1 U of FDH deposited onto the surface; a 3 min incubation time was employed at room temperature, at open circuit. A calibration study was carried out over the range 0.10–1.00 mM fructose; a linear response was obtained under these conditions and the slope was found to be 10.085 μA $mM^{-1}$, Fig. 3A(a).

In order to investigate the possibility of increasing the sensitivity of the chronoamperometric assay, the enzyme loading was increased to 4 units, on the sensor surface. Fig. 3A(b) shows the resulting calibration plot, obtained under these conditions and demonstrates a linear range of up to 1 mM, with a slope of 16.6 μA $mM^{-1}$ ± 0.4 μA $mM^{-1}$. Fig. 3B shows typical chronoamperograms obtained for standard solutions of fructose, over the range 0.10–1.00 mM; currents were measured at 20 s

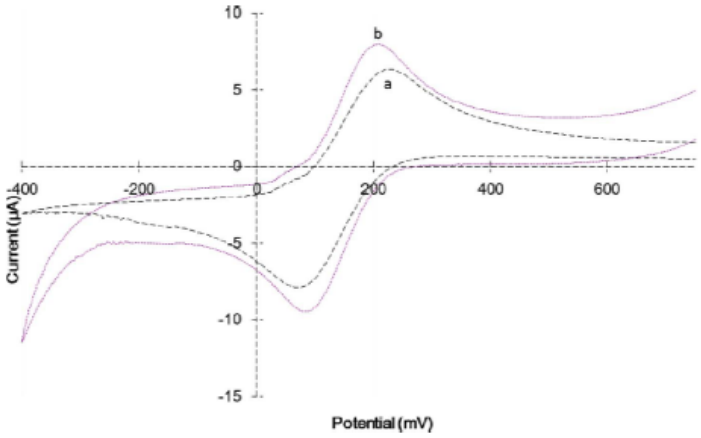


**Fig. 2.** Typical cyclic voltammograms obtained with 0.5 mM ferricyanide in 0.1 M phosphate buffer pH 7.5 containing 0.1 M potassium chloride using a scan rate of 10 mV $s^{-1}$: for (A) plain SPCE (B) SPCE-G-COOH. Voltammetric conditions: starting potential +0.8 V, switching potential −0.4 V.

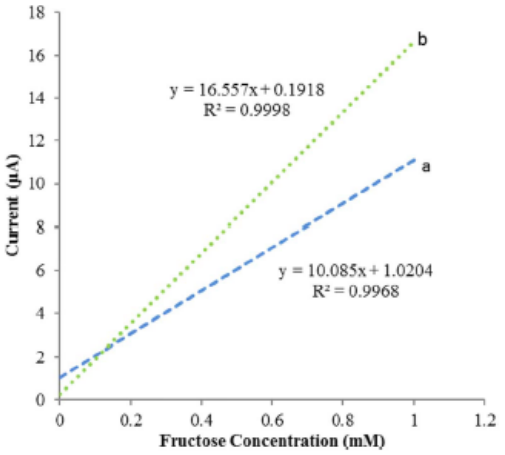


**Fig. 3A.** Calibration plots obtained for fructose using a) 1 unit of FDH with a 3 min incubation time; b) 4 units of FDH with a 3 min incubation time.

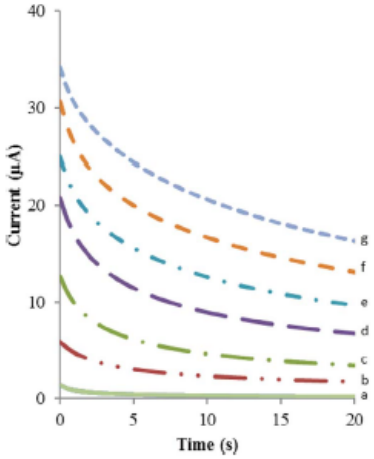


**Fig. 3B.** Chronoamperograms obtained with the fructose sensor for various concentrations of fructose: a) 0.00 mM; b) 0.10 mM; c) 0.20 mM; d) 0.40 mM; e) 0.60 mM; f) 0.80 mM; g) 1.00 mM. Sensor operation performed with 4 units of FDH with a 3 min incubation time.

after the potential was initiated. It should be mentioned that the sensitivity achieved with the COOH-G-SPCE was found to be higher than that reported in several papers. The following sensitivity values have been normalised from the original papers to give sensitivity $\mu A\ mM^{-1}\ cm^{-2}$, which allows comparison with the current assay: current study 58.56 $\mu A\ mM^{-1}\ cm^{-2}$; Biscay *et al.*, 2012 (ferrocyanide modified SPCE) 9.95 $\mu A\ mM^{-1}\ cm^{-2}$; Trivedi, Lakshminarayana, Kothari, Patel, & Panchal, 2009 (amperometric biosensor using FDH) 2.19 $\mu A\ mM^{-1}\ cm^{-2}$; Antiochia & Gorton, 2014 (osmium-polymer mediated biosensor) 1.95 $\mu A\ mM^{-1}\ cm^{-2}$. At this point it was considered that the optimised chronoamperometric assay conditions would be suitable for the analysis of a range of fruit juices; however for evaluation purposes a typical commercial apple juice was selected. This is described in the following section.

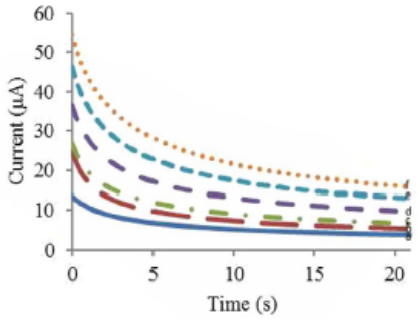


**Fig. 4A.** Typical chronoamperograms obtained with SPCE-G-COOHs for different concentrations of D-fructose: a) Apple Juice (AJ), b) 0.10 mM, c) 0.20 mM, d) 0.40 mM, e) 0.60 mM and f) 0.80 mM. Incubation time at open circuit was 180 s, followed by applied potential of + 0.3 V vs Ag/AgCl. The supporting electrolyte was 0.1 M phosphate buffer containing 0.1 M potassium chloride.

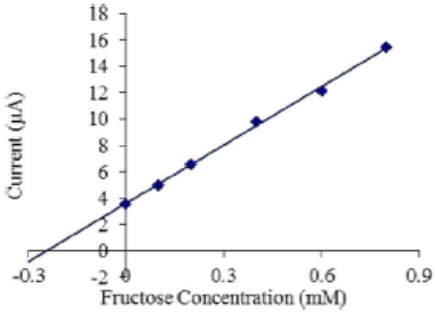


**Fig. 4B.** Typical standard addition plot, obtained using chronoamperometric currents measured 20 s after application of + 0.3 V vs Ag/AgCl.

**Table 1**
Concentration of fructose determined in dilute fruit juice and calculated original values (n = 5).

| Sample | Concentration determined after dilution (mM) | Calculated Original Fructose Concentration (mM) |
|---|---|---|
| 1 | 0.245 | 490.0 |
| 2 | 0.235 | 470.0 |
| 3 | 0.240 | 480.0 |
| 4 | 0.245 | 490.0 |
| 5 | 0.228 | 456.0 |
| Mean | 0.239 | 477.2 |
| Standard Deviation | 0.007 | 14.46 |
| CV% | 3.031 | 3.031 |

### 3.4. Analytical application

In order to evaluate the chronoamperometric assay for the measurement of fructose in commercial fruit juices, a typical apple juice product (obtained from a local supermarket) was analysed using the developed assay. Fig. 4A shows typical chronoamperometric responses obtained for the samples of apple juice, diluted 500 times (with deionized water (4A a) and after the addition of standard fructose solutions (4A b-f). Fig. 4B shows a typical standard addition plot, from which the original concentration of fructose was determined.

Table 1 shows a summary of the data obtained for the original fructose concentration of the unspiked apple juice. It should be noted

**Table 2**
Recovery of added fructose to original fruit juice sample (n = 5).

| Sample | Original Concentration (mM) | Concentration Added (mM) | Concentration Found (mM) | Fructose Recovered (mM) |
|---|---|---|---|---|
| 1 | 477.2 | 477.2 | 951.88 | 474.68 |
| 2 | 477.2 | 477.2 | 934.28 | 457.08 |
| 3 | 477.2 | 477.2 | 1010.08 | 532.88 |
| 4 | 477.2 | 477.2 | 953.79 | 476.59 |
| 5 | 477.2 | 477.2 | 992.68 | 515.48 |
| | | | Average | 491.34 |
| | | | Standard Deviation | 31.53 |
| | | | CV% | 6.42 |
| | | | Mean Recovery | 97.12% |

that column 2 refers to the concentration on the screen-printed transducer; column 3 refers to the concentration of fructose corrected for the dilution factors.

The data in the Table 1 indicates that the mean original concentration of fructose was 477.2 mM; the precision data of 3.031% suggests that the method shows promise for analysis for fructose in fruit juices.

It is known that ascorbic acid is present in apple juice of concentrations around 2.15 mM (SelfNutionData, 2014). In order to determine whether this vitamin would affect the response obtained for fructose, in the apple juice sample, a fixed concentration of 2.15 mM ascorbate was added to the neat apple juice followed by dilution (as described earlier). The resulting chronoamperograms did not show any increase in anodic current for any of the solutions shown in Table 1. This is perhaps not surprising bearing in mind the high ratio of fructose to ascorbic acid, present in the sample. This study demonstrated that ascorbic acid at the levels present in the commercial apple juice did not influence the magnitude of the fructose response; therefore no complicated sample preparation procedures were required. The recovery of fructose, added to the original apple juice sample is summarised in Table 2.

The data in Table 2 shows that the mean recovery of added fructose is 97.12% and the co-efficient of variation of 6.42%. Clearly the data shown in Tables 1 and 2 demonstrates that the chronoamperometric bioassay should give reliable data for the analysis of fructose concentration in fruit juice products.

## 4. Conclusion

This paper describes the development of a simple, low cost chronoamperometric assay, based on the electrocatalytic oxidation of FDH with a SPCE-G-COOH, and its evaluation using a commercial fruit juice. It was shown that the incorporation of modified graphite nanoparticles enhanced the sensitivity and improved the precision of the response towards the enzymatically generated ferrocyanide species, compared with a plain SPCE. The calibration studies indicated that a linear response could be obtained up to 1.00 mM fructose using a mediator concentration of 3 mM. Consequently, the proposed biosensor could be adapted for a wide range of food products. It should be mentioned that sample preparation only involves the dilution of the sample prior to analysis; which is performed only on 40 µl, of solution, applied directly onto the sensor surface. Therefore, it is readily feasible that this approach could be performed near to the point of production and used as a quality control method. It should be mentioned that the detection limit of 8 µM was achieved in the current study; however the detection limits in the commonly used Brix test has a limit of detection of 556 µM (Cejpek, 2012) and a method involving HPLC with UV–visible spectrometry had reported a detection limit of 222 µM (Bevers, Wijntje, & de Haan, 2005). Therefore, we believe that the current amperometric fructose bioassay, employing a SPCE-G-COOH, holds promise for applications where other conventional techniques do not have the desired detection limit.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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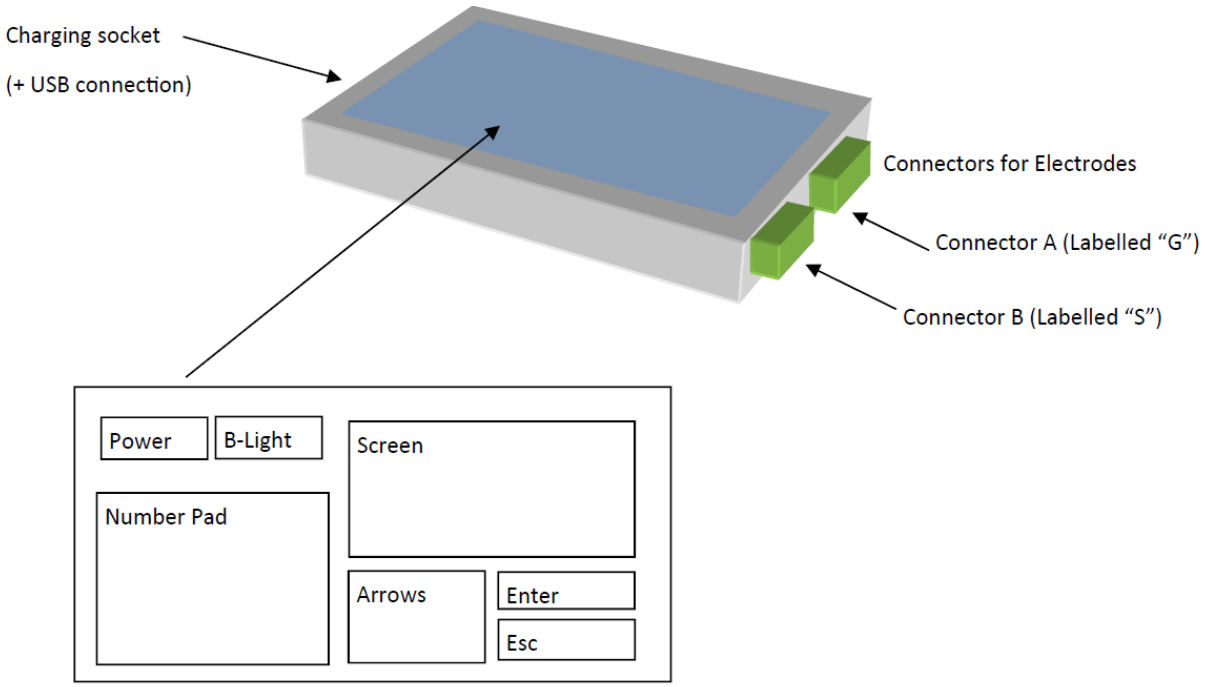
## 7.2 Original Schematic Diagram of Programming




1) OEM EmStat3 (doesn't require impedance)

2) Pre-programmed (to be used by non technical staff), each connector needs to have two different series of parameters to be entered by user via number pad (including decimal points).

3) Both connectors require a delay (open circuit) then a current reading at a set time and potential (closed circuit) both measure simultaneously

4) The reading to manipulated by parameters given in step 2, to give end result

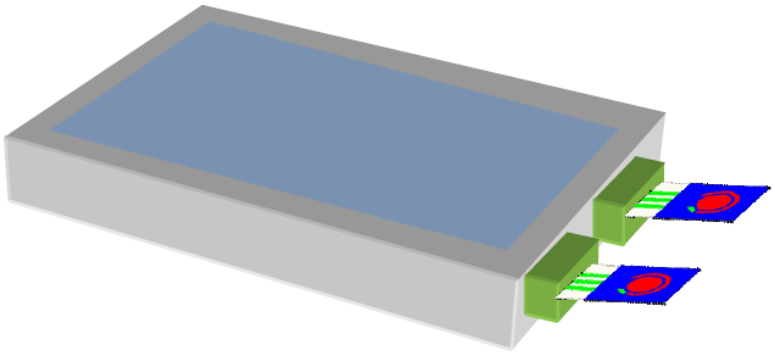
5) For Connector B results, the result is calculated from manipulation of parameters minus end result of Connector A

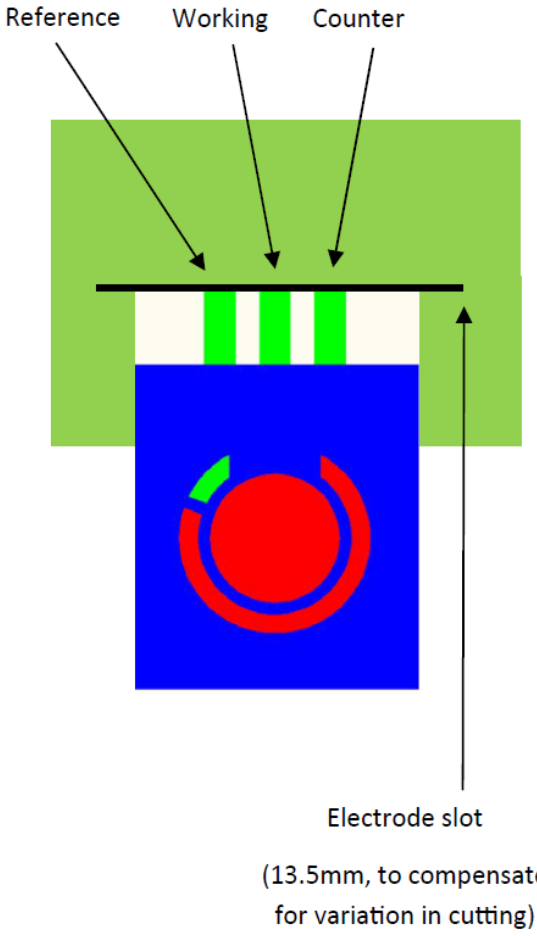
6) USB for modification of internal calculations/software

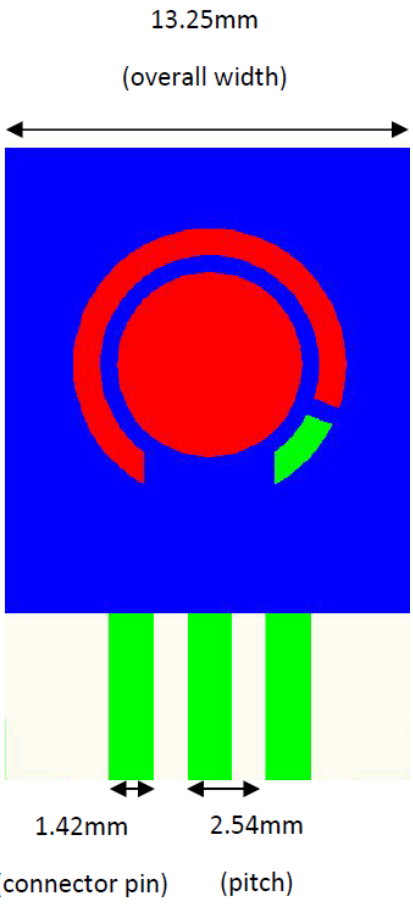
7) Charging socket needs to be compatible for different voltages (as being used worldwide), can be don't via transformer

8) Needs to be splash proof and robust for field work

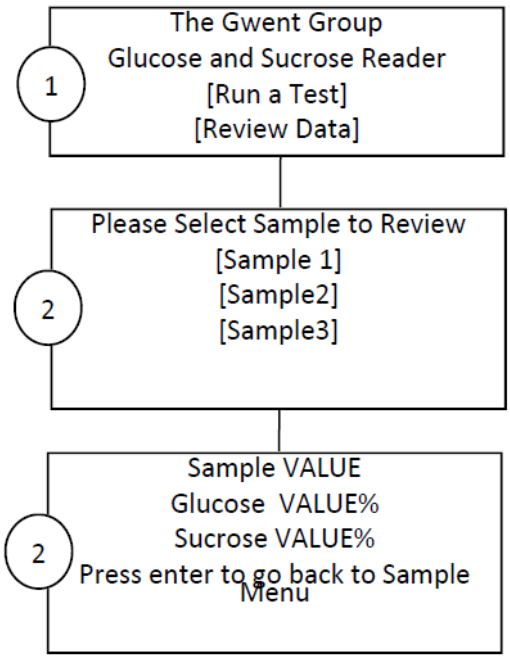
9) Current ranges = 100µA to −100µA




Reference
Working
Counter
Electrode slot
(13.5mm, to compensate
for variation in cutting)


13.25mm
(overall width)
1.42mm
(connector pin)
2.54mm
(pitch)

## Main Menu System




Screen 1

- [Run a Test] selected and enter pressed. Go to **Testing Samples Menu System screen1**
- [Review Date] selected and enter pressed. Go to **screen2**

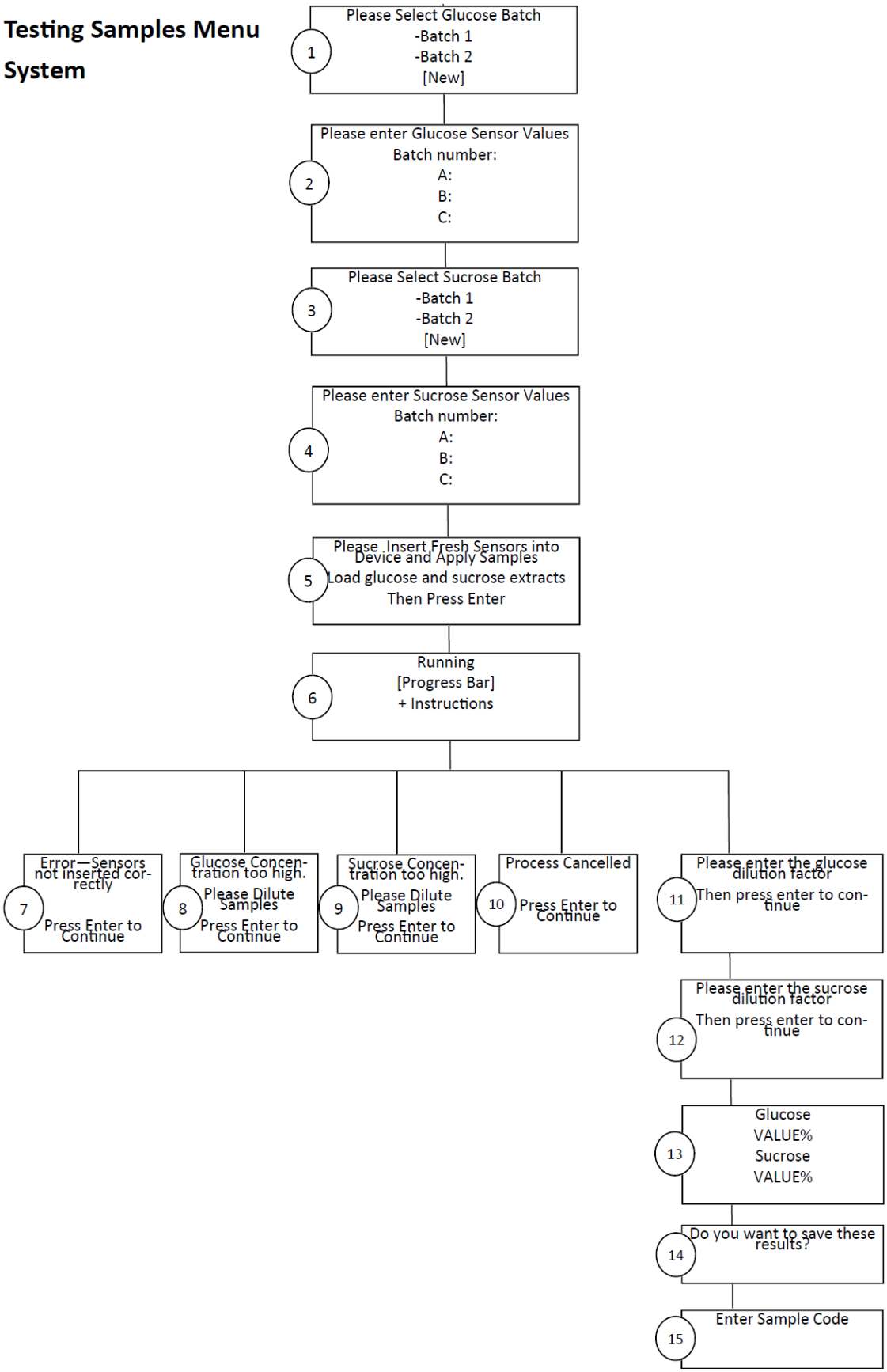
Screen 2

- [Sample x] selected. *Sample value*, *Glucose%* and *Sucrose%* recalled. Go to **screen3**
- Esc button pressed. Go to **screen1**

Screen3

- *Sample value*, *Glucose%* and *Sucrose%* shown
- Esc button pressed. Go to **screen1**

**Testing Samples Menu System**



Screen 1

- A previous batch of glucose selected. Values for $A_{glucose}$, $B_{glucose}$ and $C_{glucose}$ recalled. Go to **screen 3**
- [New] selected. Go to **screen 2**
- Esc button pressed. Go to **main menu screen1**

Screen 2

- Values for Batch number ($Batch_{glucose}$), A ($A_{glucose}$), B ($B_{glucose}$) and C ($C_{glucose}$) entered and enter pressed. Store values and add to **screen1** list. Go to **screen 3**
- Esc button pressed. Go to **screen 1**

Screen 3

- A previous batch of glucose selected. Values for $A_{glucose}$, $B_{glucose}$ and $C_{glucose}$ recalled. Go to **screen 5**
- [New] selected. Go to **screen 2**

Screen4

- Values for Batch number ($Batch_{sucrose}$), A ($A_{sucrose}$), B ($B_{sucrose}$) and C ($C_{sucrose}$) entered and enter pressed. values and add to **screen3** list. Go to **screen 5**
- Esc button pressed. Go to **screen 1**

Screen 5

- Enter pressed. Go to **screen 6**
- Esc button pressed. Go to **screen 1**

Screen 6

Progress Bar shown, instructions shown

0-20 seconds - "Please stir Glucose Sensor"

21-30 seconds - "Please stir Sucrose Sensor"

31-224 seconds - "Please wait while sample is being analysed"

- If $i_{glucose}$ and/or $i_{sucrose}$ is less than 0.4μA (value maybe changed) then go to **screen 7**
- If $i_{glucose}$ is more than $C_{glucose}$ then go to **screen 8**
- If $i_{glucose}$ is less than $C_{glucose}$ and $i_{sucrose}$ is more than $C_{sucrose}$ then go to **screen 8**
- If $i_{glucose}$ is less than $C_{glucose}$ and $i_{sucorse}$ is less than $C_{scurose}$ then go to **screen11**
- Esc button pressed. Go to **screen1**

Screen7

- Enter pressed. Go to **screen1**
- Esc button pressed. Go to **screen1**

Screen 8

- Enter pressed. Go to **screen1**
- Esc button pressed. Go to **screen1**

Screen 9

- Enter pressed. Go to **screen1**
- Esc button pressed. Go to **screen1**
-

Screen 10

- Enter pressed. Go to **screen1**
- Esc button pressed. Go to **screen1**

Screen 11

- Value entered ($D_{glucose}$). Followed by enter button. Go to **screen 12**

Screen 12

- Value entered ($D_{scurose}$). Followed by enter button. Go to **screen 13**

Screen 13

- Calculate glucose concentration ($Conc_{glucose}$). $Conc_{glucose} = ((i_{glucose} — B_{glucose})/A_{glucose})*D_{glucose}$
- Calculate sucrose concentration ($Conc_{sucrose}$). $Conc_{sucrose} = ((i_{sucrose} — B_{sucrose})/A_{sucrose})*D_{sucrose}$
- Calculate glucose percentage (Glucose%) - formula to be decided
- Calculate sucrose percentage (Sucrose%)- formula to be decided
- Show "Glucose = *Glucose%*" and "Sucrose = *Sucrose%*"
- Enter pressed. Go to **screen14**

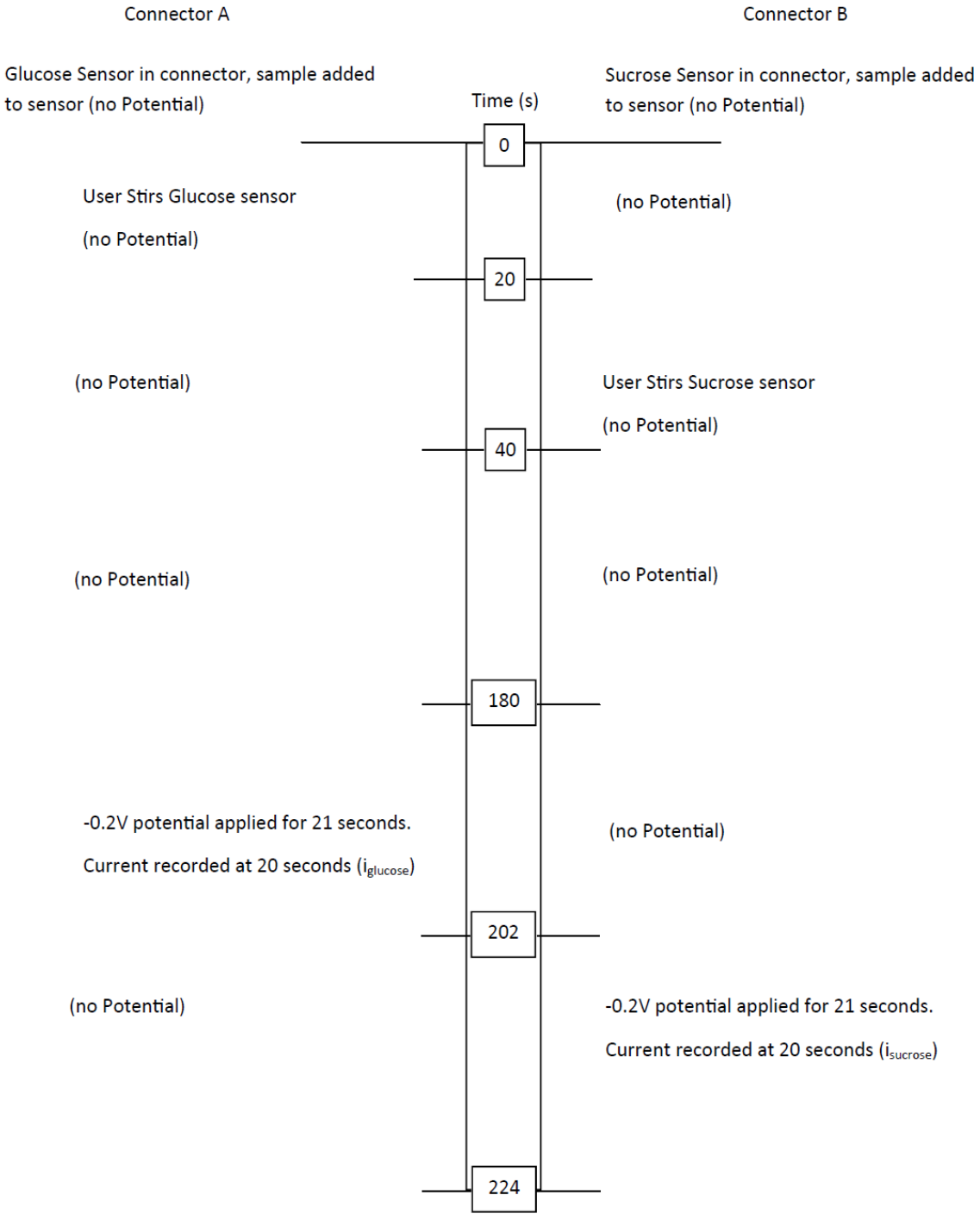
Screens 14

- Enter pressed. Go to **screen15**
- Esc button pressed. Go to **screen1**

Screen 15

- Sample code (Sample value) entered. Enter pressed. Store *Sample value*, *Glucose%* and *Sucrose%*, add to menu list in **main menu screen2**. Go to **screen1**

## Time schedule during Testing (Testing Samples Menu System screen6)



## 7.3 Technical Data Sheet for Glucose, Sucrose and Fructose Biosensors

## SunSens Biosensor System
***For Processed Potatoes***

**From the SunSens family from SunChemical**

### 1. Description

Sun Chemical has produced a range of biosensors, aimed towards the agricultural market. The biosensors are designed to work with the SunChemical Potentiostat and the appropriate android application.

The android application is supplied on a preloaded tablet. Each biosensor works with its own unique application.

Potentiostat Demonstration Video
Potentiostat Information Video


### 2. Potentiostat & Application features

- Step-by-step on screen guidance
- Bluetooth connectivity between tablet and potentiostat, making the device portable and easy to handle
- Data storage of tests, including a date-stamp and personalised sample labelling, onto the tablet. Easily transferred to a PC
- Self sensor calibration carried out with QR-code scanning, using the tablet's front camera. Avoiding the user from carrying out calibrations and saving time
- Progress bar, showing the overall status of the measurement
- The software can be modified to display the data in the units desired by the user (modification of software carried out by SunChemical).
- The application converts the concentration to percentage, adjusting accordingly with the dry matter percentage (manual input by the user, an assumption of 22.5% dry matter is also an option).

### 3. Product Range

#### 3.1 BIO-GLU-A1 Low Level Potato Glucose Biosensors

The product provides a simple and rapid approach to measuring low level glucose, in potatoes. The measurement is carried out in conjunction with the Sun Chemical Potentiostat, which guides the user through each step of the process. Each sensor is a single shot disposable biosensor.

The system comes with a sample preparation manual, providing the user with the best results.

The sensors have a range of 0.010mM to 0.600mM. Higher concentrations can be measured using a dilution step (software adjusts the end value according to the dilution factor).

Each pot of contains 20 sensors, package with desiccant.

*Benefits*

- 40 µl of test sample is require per test.
- Can be used with or without the Low Level Potato Sucrose Biosensor (BIO-SUC-A1).
- Requires Sun Chemical Potentiostat and Tablet.
- Each test (from applying test solution to strip) has a measurement time of 3 - 4 minutes.

### 3.2 BIO-SUC-A1 Low Level Potato Sucrose Biosensors

The product provides a simple and rapid approach to measuring low level sucrose, in potatoes. The measurement is carried out in conjunction with the Sun Chemical Potentiostat, which guides the user through each step of the process. Each sensor is a single shot disposable biosensor.

The system comes with a sample preparation manual, providing the user with the best results.

The sensors have a range of 0.010mM to 0.600mM. Higher concentrations can be measured using a dilution step (software adjusts the end value according to the dilution factor).

Each pot of contains 20 sensors, package with desiccant.

*Benefits*

- 40 µl of test sample is require per test.
- **Must be used in conjunction** with the Low Level Glucose Biosensor (BIO-GLU-A1).
- Requires Sun Chemical Potentiostat and Tablet.
- Each test (from applying test solution to strip) has a measurement time of 3 - 4 minutes.

### 3.2 BIO-FRU-A1 Low Level Potato Fructose Biosensors

The product provides a simple and rapid approach to measuring low level fructose, in potatoes. The measurement is carried out in conjunction with the Sun Chemical Potentiostat, which guides the user through each step of the process. This product is a one shot disposable dual biosensor.

The system comes with a sample preparation manual, providing the user with the best results.

The sensors have a range of 0.025mM to 0.800mM. Higher concentrations can be measured using a dilution step (software adjusts the end value according to the dilution factor).

Each pot of contains 20 sensors, package with desiccant.

*Benefits*

- 40 µl of test sample is require per test.
- Requires Sun Chemical Potentiostat and Tablet.
- Each test (from applying test solution to strip) has a measurement time of 3 - 4 minutes.

## 4. General Handling

### 4.1 Storage and Shipping

For long time storage - keep between 1 – 10°C / 34 – 50°F. Sensors should be stored in the container provided, ensuring the lid is securely closed.

When in usage – keep container out of direct sunlight. The container can be at room temperature for 3 hours (for every 24 hours). Sensors should be stored in the container provided, ensuring the lid is securely closed between removal of sensors.

### 4.2 Waste disposal

This should be carried out in accordance with good industrial practice, observing all the appropriate regulations and guidelines.

### 4.3 Manual Handling

Gloves should be used when handling the sensors. Avoid contact with the operational area (defined by the mesh).

## 5. Processing for Produce

SunChemical offers two approaches to the extraction of potato juice.

- For manual extraction, SunChemical can provide a manual powered juice extractor, which can then be used on the sensors.
- For electronic extraction, SunChemical can provide a hand-held blender

Both techniques come with protocols and manuals, allowing the user to obtain the best results.

*The application can be adapted to accommodate pre-existing protocols, already carried out by the user.*

## 6. Disclaimers

This information has been carefully compiled from experience gained in field conditions and extensive laboratory testing. However, the products' performance and its' suitability for the customers' purpose depend on the particular conditions of use and the material being tested. We recommend that customers satisfy themselves that each product meets their requirements in all respects before commencing a production run. Since we cannot anticipate or control the conditions under which our products are used, it is impossible to guarantee their performance. All sales are also subject to our standard terms and conditions.

## 7. Technical Assistance / Contacts

Sun Chemical are an international company, and as such can offer technical, engineering and sales support to our customers worldwide.

For further information regarding this product, or any of our extensive range of materials please contact your local Sun Chemical team or visit the Technical Help Desk at website:
http://www.sunchemicalhelpdesk.com

## 7.4 Glucose and Sucrose Potentiostat Manual

SunChemical®

a member of the DIC group

Color & Comfort

# MANUAL FOR THE SUNSENS POTENTIOSTAT

## FOR GLUCOSE AND SUCROSE MEASUREMENTS

This document is intended to provide information about:

1. Parts and Components
2. Charging and Operating the SunSens Potentiostat
3. Prior Setup to Testing Extracts
4. Running the Application and Testing
5. Reviewing Data
6. Trouble Shooting
7. Copying and Reviewing the Data using a PC

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## 1. Parts and Components

Each instrument is provided with a:

- SunSens Potentiostat
- SunSens Glucose and Sucrose Connector Module
- Stirrer
- 90° USB and multi adapter charger for the SunSens Potentiostat
- Tablet (with pre-installed Glucose and Sucrose application)
- USB (and UK Plug) charger for the Tablet

Users will require:

- Tweezers
- Micro-pipette tips
- Lint-free tissue
- Gloves
- Sample Preparation Tools
- Micro-Pipette (able to deposit 40µl)

(Model of tablet may vary)



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## 2. Charging and Operating the SunSens Potentiostat

The SunSens Potentiostat is a battery operated, stand-alone device, with an internal battery. For initial use or when the red battery light is flashing, the device can be recharged using the 90° USB cable.

The device can be charged during measurement operation. This is achieved by attaching the module to the device, after the USB cable has been plugged in.

To turn on the SunSens Potentiostat, hold down the centre power button until a white light appears around the button. *The blue-tooth light will flash until the device is connected to a tablet.*

If the blue-tooth light is not on, the press the centre power button.

### 3. Prior Setup to Testing Extracts

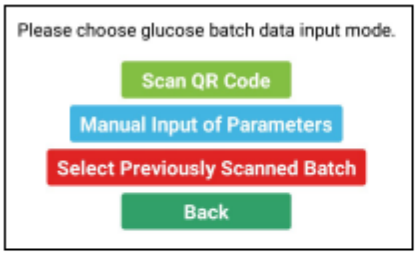
*Prior to running a test, the extract sample should be prepared according to the procedure.*

### 4. Running the Application and Testing

Once the tablet is turned on, click the SunSens Glucose and Sucrose Application Icon. This can be found on the front page of the tablet.


Once the application is open, a screen containing a "Run a new test" button should appear. Click the button to run a new test.


*NB. This page only contains one button, when the application has been run for the first time or when all the review data has been deleted.*




When a new test is run, the application will ask for the batch information of the Glucose sensor. These values will change from batch to batch.

When testing, using a new batch of Glucose sensors, press the "Scan QR Code" button.

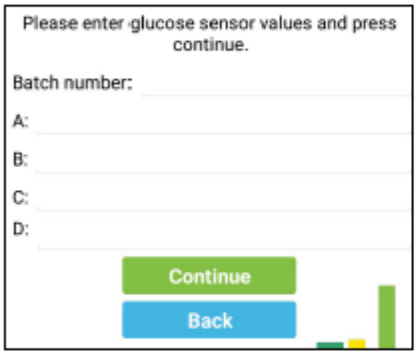
***Scanning QR-Code***

Once the "Scan QR Code" button is pressed, the front camera of the tablet will be activated.
Select the batch of Glucose sensors (Product Code BIO-GLU-A1) that is to be used for testing,
Hold the QR Code steadily above the front of the tablet, the QR Code is located on the top side of the vials. For best results the QR code should fill most of the centre area of the screen.

*NB. If the incorrect product is being scanned then and message "Incorrect Product" will appear.*

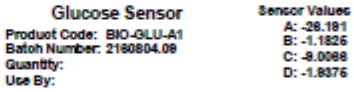

***Manual Input of Parameters***

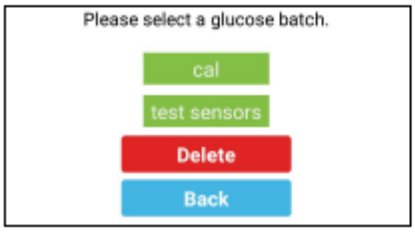
If the "Manual Input of Parameter" is selected, a new screen will appear. This approach should only be used in the event that the QR-Code has been damaged.




When entering the batch values of the sensors, care should be taken as the values contain negative numbers of up to five significant figures.

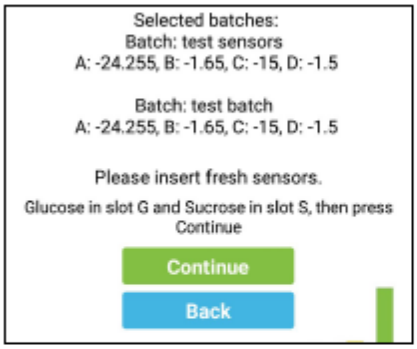
The batch values can be found on each pot of sensors.




*Selecting a Previously Scanned Batch*

If the "Select Previously Scanned Batch" is selected, a new screen will appear. Previously scanned batches will appear as buttons. Ensure that the Batch number on the screen is the same as the Batch number of the pot that's being used.




Repeat the process for the Sucrose Sensors

A summary screen of the sensors parameters will appear. It is recommended to check these values against the values that appear on the main label of the sensors.

During this screen the sensors should be inserted into the device.

During this stage, ensure that the module is connected securely onto the SunSens Potentiostat.



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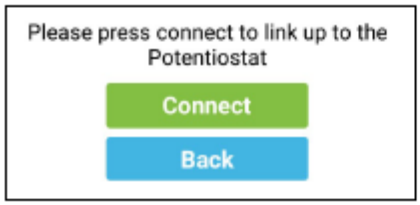
Using a tweezers and gloves (not provided), remove the sensors from their container and place in the module.

*NB. Do not touch the central circle section of the sensor*

The glucose sensor should be inserted into slot "S" and the sucrose sensor should be inserted into slot "G".

*NB. Ensure that the sensors are inserted correctly, with the connectors aligning to the tracks of the sensors*

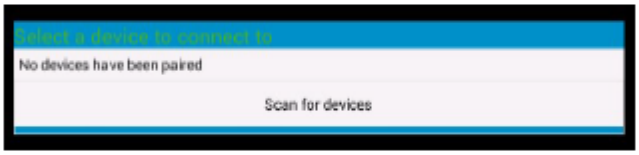
Once the sensors have been inserted correctly, press the "continue" button.




The application will ask you to connect the Tablet to the SunSens Potentiostat. Ensure the potentiostat is turned on (see section 2) and press "Connect".

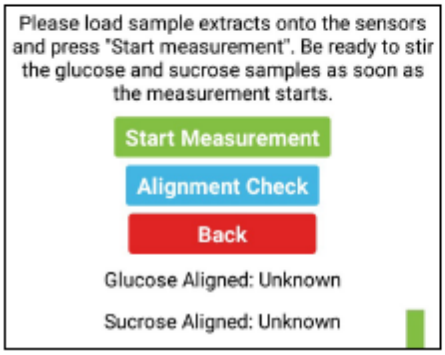
If the tablet has been used with the potentiostat before then it will appear in the list and should be selected. This will re-initialise the connection automatically.

If the tablet has not been used with the potentiostat before then a dialog box will appear, with "No devices have been paired". In this instance press the "Scan for devices" button.



Select the description beginning with "PS-".


The device may ask you for a PIN, if it does, type "1234" and press "OK"

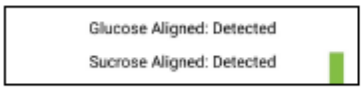



The application will then ask the user to load the extract onto the sensors.

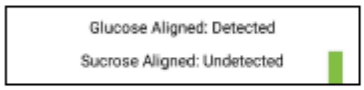
40µl of the glucose extract should be deposited onto the Glucose sensor (slot G).
40µl of the sucrose extract should be deposited onto the Sucrose sensor (slot S).

To check that the sensors are positioned correctly, press the "Alignment" button

*NB. The extract requires depositing on the sensors before pressing the "Alignment" button.*



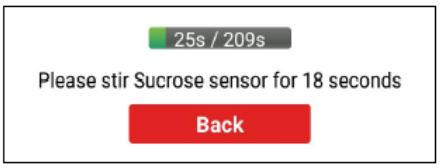

If the sensors are correctly aligned the screen will display them as "Detected"




If one of the sensors is not aligned correctly, or not inserted, then "Undetected" will be displayed.

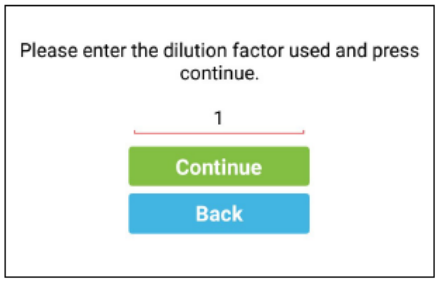

Once the "Start" button has been pressed a progress bar will appear, with the instructions to stir the Glucose A sensor. During this time the Glucose A sensor should be stirred, for 20 seconds, using the stirrer provided.


A sound will occur when it is time to stop stirring. The stirrer will then require a simple wipe using a lint free tissue.




The application will give instructions to stir the sucrose sensor, for 20 seconds.


A sound will occur when it is time to stop stirring. The stirrer will then require a simple wipe using a lint free tissue.



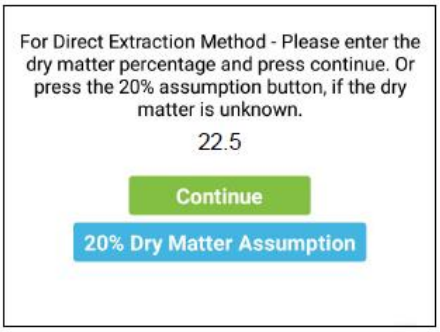

Once the extract has been analysed, the application will ask for the extraction dilution factor. If the extract has not been further diluted, from the protocol, enter "1".

If the extract has been diluted further, then the number of parts of water to the number of parts of extract requires to be added together.

*Example: 9 parts water with 1 part extract will result in a 10 extract dilution factor.*

Press "Continue" once the extract dilution factor has been entered.

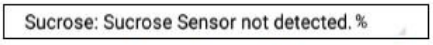

Select the "Use Direct Extraction Method" option



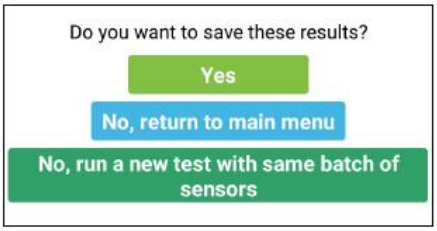

If a dry matter analysis has been carried out, enter the dry matter (in percentage), and press continue.

If the dry matter is unknown, then press the "20% Dry Matter Assumption" button. This will use 20% as the dry matter percentage.


The results will be displayed as a percentage. Select the small grey arrow to convert to alternative units. Press "Continue" to continue to the next page.

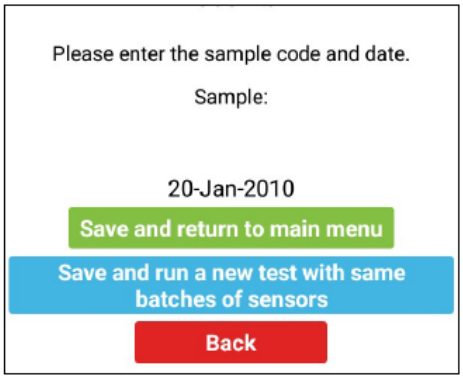



*NB. If the sucrose sensor is not present then "Sucrose Sensor not detected" will be presented as the sucrose result.*




The application will ask the user if they wish to save the results.

- Press "Yes" to save the results
- Press "No" to not to save results and to go back to the main menu.
- Press "No, run a new test with the same batch of sensors" if the user does not want to save but does want to run another test using the same scanned pots of sensors.



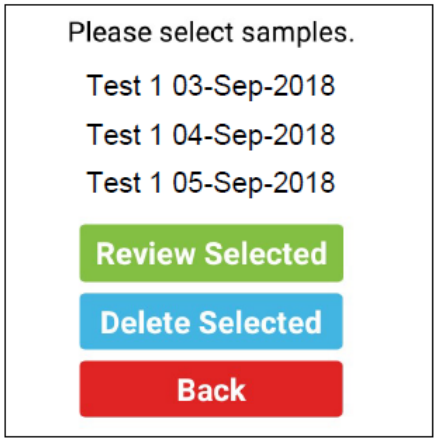

If the user has selected "yes", to save the results, a new screen will appear. The user will be asked to enter a suitable name for the sample. The date will automatically be added to the sample name.

The user can press "Save and return to main menu", to save the data and return back to the main menu.

Alternatively, the user can press "Save and run a new test with the same batches of sensors. This will allow the user to run another test using the same scanned pots of sensors, without having to input the sensor details again.

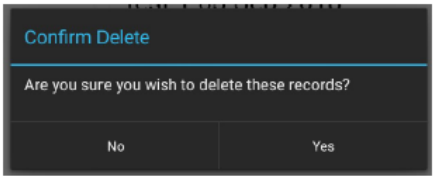
## 5. Reviewing Data


If data has been saved onto the tablet, then a "Review data" button will appear on the opening page of the application. Press this button to review the data.




The data will be shown in the format the user had previously saved the data as. The user can select multiple samples to review. This is achieved by ticking the samples and then pressing "Review Selected". This will show the samples that were selected, with the results.

If the user requires deleting samples, then tick the samples that are desired to be deleted followed by pressing the "Delete Selected".




A confirmation dialog box will appear, in case the button is accidentally pressed.

## 6. Trouble Shooting

| Error Message | Cause |
|---|---|
| Sample less than limits of detection<br>Glucose: Sample less than limits of detection. Less than 0.0004 | The glucose quantity is less than the limits of detection. This may occur with over diluting the extract. |
| Glucose dilution required, suggested 1 part solution to [x] part(s) water<br>Glucose dilution required, suggested dilution of 3 times. | The glucose quantity is more than the limits of detection. The extract requires diluting, the software will provide a suggested dilution but this is only an approximation |
| Glucose sensor not detected. Please check alignment<br>Error - Glucose Sensor not detected. Please check alignment | The glucose sensor has not been detected by the potentiostat. Ensure that a glucose sensor is inserted and aligned correctly |
| Sucrose Sensor not detected<br>Sucrose: Sucrose Sensor not detected. % | The sucrose sensor has not been detected by the potentiostat. Ensure that a sucrose sensor is inserted and aligned correctly.<br>*NB. This will occur if only the glucose is being measured* |
| Sucrose sample less that limits of detection<br>Sucrose: Sucrose sample less than limits of detection. | The glucose quantity is less than the limits of detection. This may occur with over diluting the extract. |
| Incorrect Product Scanned | The incorrect pot of sensors has been scanned. Check to ensure that the correct pot of sensors is being scanned. |



## 7. Copying and Reviewing the Data using a PC

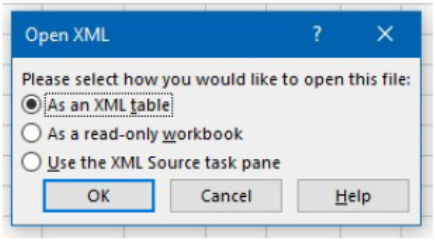
There are various ways of copying the data to a PC. The method below will explain how to copy the data using the USB cable provided and importing to excel.

Connect the tablet to a suitable windows based PC. When connected the tablet will appear as a portable device, in "Computer"/"My Computer".

Load Office Excel and select "open"
Select the file from the location:

*This PC\Galaxy Tab A (2016)\Tablet\Sun\Gands1-0\Samples.xml.*

Excel will present the following dialog box:




Select "OK"

The information in each column is described below:

| | |
|---|---|
| A | Sample Code |
| B to H | Glucose Sensor Batch Information |
| I to O | Sucrose Sensor Batch Information |
| P | Glucose Percentage Result |
| Q | Sucrose Percentage Result |
| R to T | This information is for diagnostic purposes only |