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.

Keywords: Fructose, fructose dehydrogenase, chronoamperometric, screen-printed, graphite, nanoparticles

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 (Guillaume, Delobel, Sablayrolles and Blondin, 2007; Bauer and Pretorious, 2000). 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 (Biscay, Rama, Garcia, Reviejo, Carrazón and García, 2012; Tsujimura, Nishina, Kamitaka and Kano, 2009; Antiochia and Gorton, 2014). One of the current methods, of determining fructose and other simple sugars, involves the °Brix test (Cejpek 2012; Kawahigashi, Kasuga, Okuizumi and 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 and Shaligramca, 2015). An alternative approach is based on fourier transform infrared spectroscopy (Reru, Wibowo and Rondonuwu, 2016; Wang, et al., 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, Westmacott, Honeychurch, Crew, Pemberton and Hart, 2016; Hughes, Pemberton, Fielden and Hart,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 and Hart, 2016; Kanyong, Pemberton, Jackson and 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 and Hart, 2015). 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 nano-material 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 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], 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

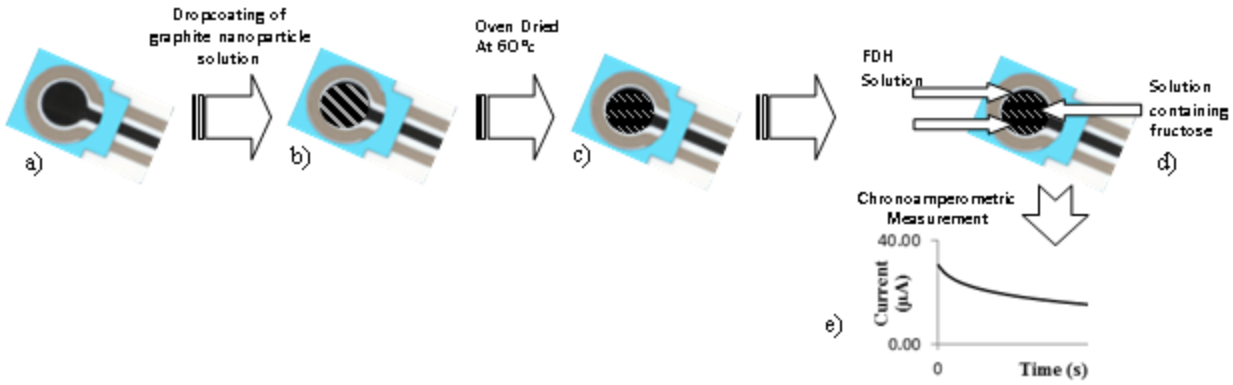


Fig.1. Scheme showing the fabrication of the fructose biosensor and chronoamperometric measurement of fructose: a) Plain SPCE; b) SPCE with deposition of nanoparticles in solution; c) SPCE with dried nanoparticles; d) addition of 10 µl FDH, 10µl ferricyanide; 20 µl of solution containing fructose; e)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

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 0.5mM 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.

2.4 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.

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

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

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

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

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.

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.

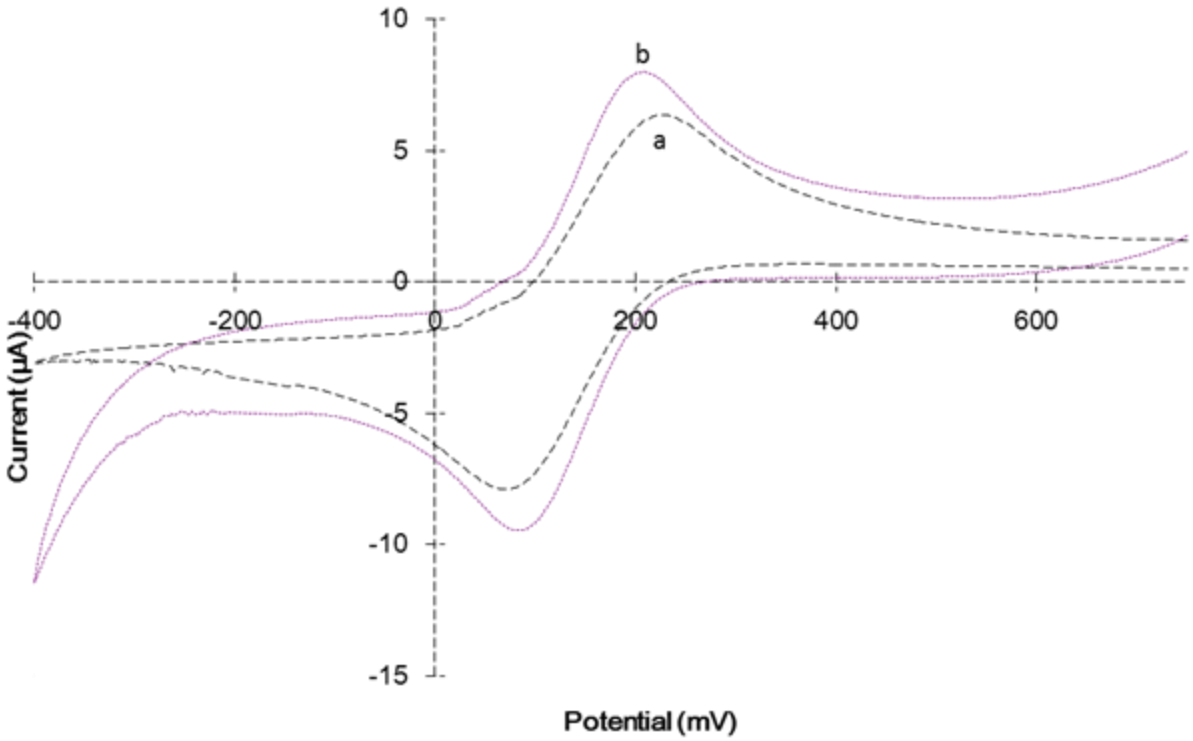


Fig.2. A typical cyclic voltammograms obtained with 0.5 mM ferricyanide in 0.1 M phosphate buffer pH 7.5 containing 0.1 M potassium chloride using a scan rate of 10 mV s^{-1}: for (A) plain SPCE (B) SPCE-G-COOH. Voltammetric conditions: starting potential +0.8 V, switching potential -0.4 V.

Fig.2 also shows that there is a difference in the peak separation (ΔE_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

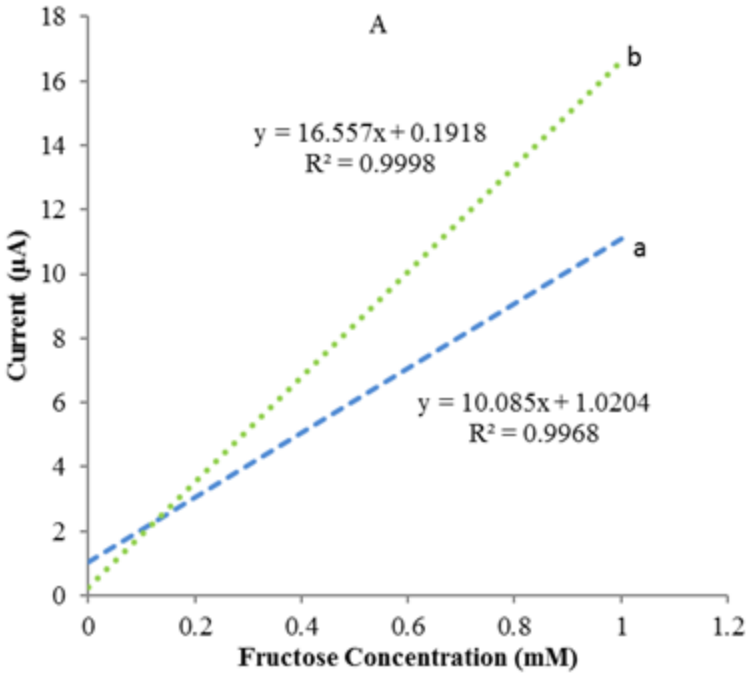
redox reaction is quasi-reversible; however, the reaction at the SPCE-G-COOH appears to be more favourable. Further evidence for this is obtained the charged transfer coefficient (α), using equation 4 (Kirsch, Hart, Bird, Luxton and McCalley, 2001) where is n is the number of electrons involved in the rate determining step.

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

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 and 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 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.3A(a).



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

177 In order to investigate the possibility of increasing the sensitivity of the chronoamperometric assay, the enzyme
178 loading was increased to 4 units, on the sensor surface. Fig.3A(b) shows the resulting calibration plot, obtained
179 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}.
180 Fig.3B shows typical chronoampergrams obtained for standard solutions of fructose, over the range 0.10 mM to 1.00
181 mM; currents were measured at 20 seconds after the potential was initiated. It should be mentioned that the
182 sensitivity achieved with the COOH-G-SPCE was found to be higher than that reported in several papers. The
183 following sensitivity values have been normalised from the original papers to give sensitivity µA mM^{-1} cm^{-2}, which
184 allows comparison with the current assay: Nicholas, et al. 2017 (current study) 58.56 µA mM^{-1} cm^{-2}; Biscay, Rama,
185 García, Reviejo, Carrazón, García, 2012 (ferrocyanide modified SPCE) 9.95 µA mM^{-1} cm^{-2}; Trivedi, et al. 2009
186 (amperometric biosensor using FDH) 2.19 µA mM^{-1} cm^{-2}; Antiochia, et al., 2014 (osmium-polymer mediated
187 biosensor) 1.95 µA mM^{-1} cm^{-2}. At this point it was considered that the optimised chronoamperometric assay
188 conditions would be suitable for the analysis of a range of fruit juices; however for evaluation purposes a typical
189 commercial apple juice was selected. This is described in the following section.

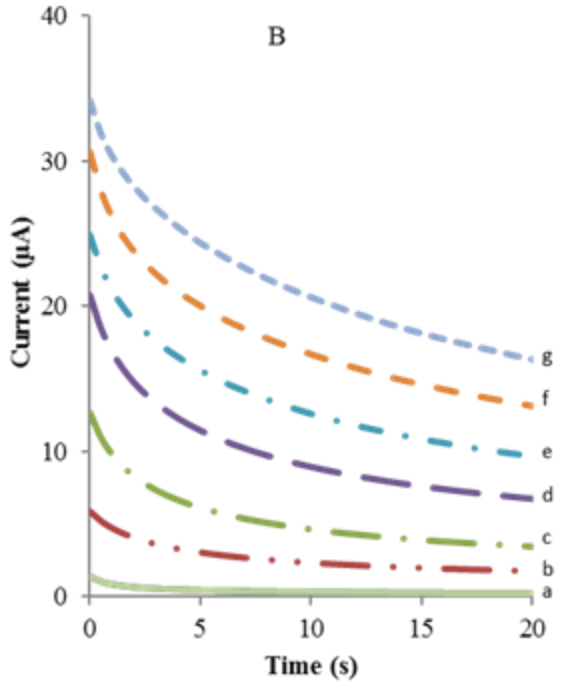

Fig. 3B Cchronoamperograms obtained with the fructose sensor for various concentrations of fructose: a) 0.00 mM; b)0.10 mM; c)0.20 mM; d)0.40 mM; e)0.60 mM; f)0.80 mM; g)1.00 mM. Sensor operation performed with 4 units of FDH with a 3 minute incubation time.

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 deionised 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.

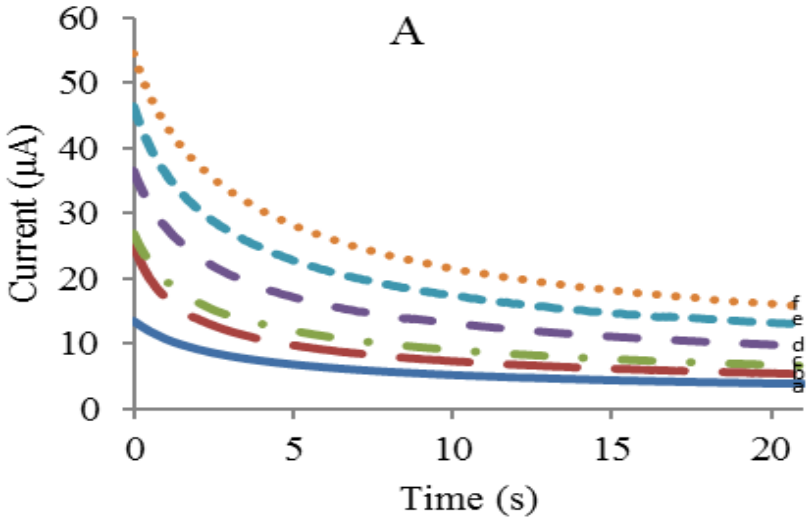

Fig.4A. Typical chronoamperograms obtained with SPCE-G-COOHs for different concentrations of D-fructose: a)Apple Juice(AJ), b)0.10 mM, c)0.20 mM, d)0.40 mM, e)0.60 mM and f)0.80 mM. Incubation time at open circuit was 180 s, followed by applied potential of + 0.3 V vs Ag/AgCl. The supporting electrolyte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

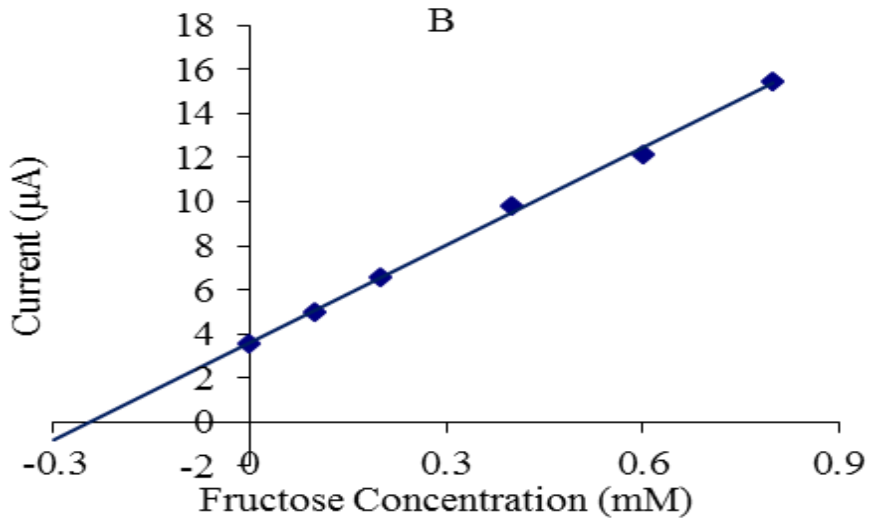

Fig.4B. Typical standard addition plot, obtained using chronoamperometric currents measured 20 s after application of +0.3 V vs Ag/AgCl

Table 1 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.

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

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 (SELFNutrition Data, 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.

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 %

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 Table 1 and Table 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 HAJM, Wijntje R and de Haan AB 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.

Captions

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, 10µl ferricyanide; 20 µl of solution containing fructose; e) Chronoamperometric measurement

Fig.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. 3A 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.

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 minute incubation time.

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

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