1 **DEVELOPMENT OF A SIMPLE, LOW COST CHRONOAMPEROMETRIC ASSAY FOR**

2 **FRUCTOSE BASED ON A COMMERCIAL GRAPHITE-NANOPARTICLE MODIFIED**

3 **SCREEN-PRINTED CARBON ELECTRODE.**

4 Phil Nicholas[1], Robin Pittson[1] and John P Hart[2*]

5 [1]The Gwent Group, Monmouth House, Mamhilad Park, Pontypool, NP4 0HZ, United Kingdom. +44-1495-750505.

6 philip@gwent.org

7 [2] Centre for Research in Biosciences, Faculty of Health and Applied Sciences, University of the West of England,

8 Bristol, Coldharbour Lane, Bristol BS16 1QY, UK

9 [*] Correspondence: john.hart@uwe.ac.uk. Tel.: +44-11-7328-2469

10 **ABSTRACT**

11 This paper describes the development of a simple, low cost chronoamperometric assay, for the measurement of

12 fructose, using a graphite-nanoparticle modified screen-printed electrode (SPCE-G-COOH). Cyclic voltammetry

13 showed that the response of the SPCE-G-COOH enhanced the sensitivity and precision, towards the enzymatically

14 generated ferrocyanide species, over a plain SPCE; therefore the former was employed in subsequent studies.

15 Calibration studies were carried out using chronoamperometry with a 40 µl mixture containing fructose, mediator

16 and FDH, deposited onto the SPCE-G-COOH. The response was linear from 0.1 mM to 1.0 mM. A commercial

17 fruit juice sample was analysed using the developed assay and the fructose concentration was calculated to be 477

18 mM with a precision of 3.03 % (n=5). Following fortification (477 mM fructose) the mean recovery was found to be

19 97.12 % with a coefficient of variation of 6.42 % (n=5); consequently, the method holds promise for the analysis of

20 commercial fruit juices.

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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

75 terephthalate polymers; Cadillac Plastics Swindon, UK). The diameter (6 mm) of the working electrode was defined

76 using a dielectric ink (GEM Product Code: D2070423P5) a concentric silver/silver-chloride served as the

77 counter/reference electrode, (GEM Electrode Design: BE2110916D1). For further studies, the surface of the

78 working electrode was modified by addition of 10µl of graphite-nanoparticles (1.787 mg ml^{-1}) (GEM code:

79 C2131210D1).

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81 The working and reference electrodes were connected to the potentiostat with GEM electrode connector (GEM

82 Code: CON002). All electrochemical studies were performed using an AutoLab [µAutoLab Type II], with General-

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

84 Fig.1 summarises the fabrication and operation of the fructose biosensor
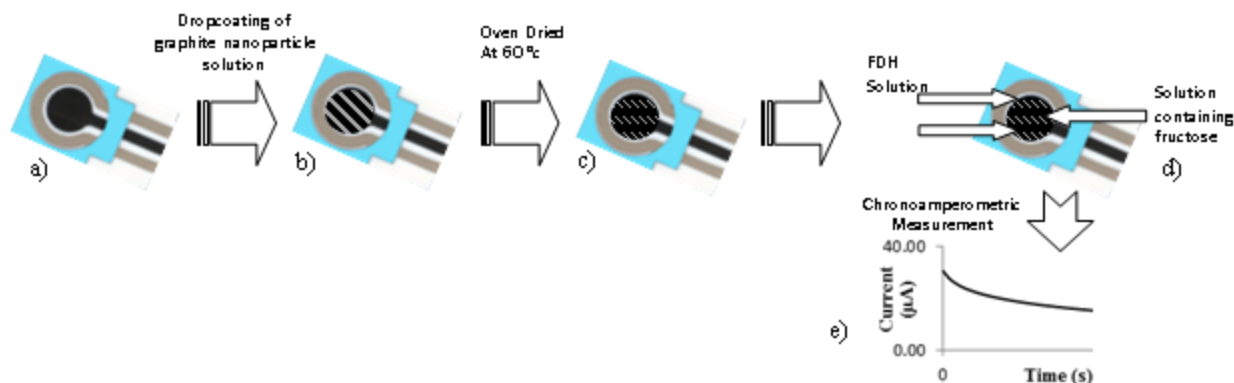
85
86 *Fig.1. Scheme showing the fabrication of the fructose biosensor and chronoamperometric measurement of fructose:*

87 *a) Plain SPCE; b) SPCE with deposition of nanoparticles in solution; c) SPCE with dried nanoparticles; d) addition*

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

89 It should be noted that during the fabrication of the nano-particle modified electrodes, the deposited nano-particles

90 were confined to the working area by the hydrophilicity of the carbon and the hydrophobicity of the underlying

91 valox substrate. This ensured that the working area remained the same between the unmodified and modified

92 working electrodes and was confirmed by visual inspections.

93 **2.3 Procedures**

94   Cyclic voltammetry was performed by depositing a 300 µl aliquot of 0.5mM ferriycanide, in 0.1 M phosphate buffer

95   pH 7.5 containing 0.1 M potassium chloride onto the surface of the screen-printed carbon electrodes. Cyclic

96   voltammetry was performed using the following conditions: initial and final potential +0.8 V; switching potential –

97   0.4 V; scan rate 10 mV s^{-1}. Potential held at +0.8 V for 20 seconds before initial cycle.

98   Calibration studies were performed using chronoamperometry with standard solutions of fructose, over the

99   concentration range 0.20 mM to 32.00 mM, in water; FDH was dissolved in McIlvaine buffer to produce

100   concentrations of either 50 U ml^{-1} or 200 U ml^{-1}. The measurement procedure involved the deposition of 20 µl of

101   either enzyme solution, onto the screen printed transducer, followed by 10 µl of 12 mM ferricyanide and 10 µl

102   fructose standard. Following an incubation time of 180 s (open circuit), with initial 20 s of agitation, the potential

103   was stepped from open circuit to +0.3 V vs Ag/AgCl. Currents were measured 20 s after application of the voltage

104   and these values were used to plot calibration graphs.

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2.4 Analytical Application

107   A preliminary study was performed with the commercial apple juice, to deduce an appropriate dilution procedure. A

108   series of dilute apple juice solutions were prepared by mixing the neat sample with deionized water to produce final

109   dilutions in the range of 1/2 and 1/512, of the original concentration. The analysis was carried out using

110   chronoamperometry as described above, and from the results the optimum dilution that produced a signal within the

111   linear range was deduced.

112   The method of standard addition was performed with the optimum dilution of the apple juice (with deionized water).

113   This was achieved by mixing the diluted apple juice with different concentration fructose standards, so that the final

114   concentration of the standard added was between 0.1 and 0.8 mM. This procedure was repeated 5 times, with each

115   data point being replicated 5 times. The concentration of fructose in the original sample was obtained from this data,

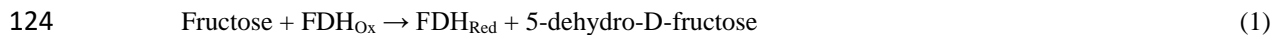116   together with the precision of the measurements.

117   The recovery of added fructose was ascertained by spiking the original sample with 477.2 mM of fructose (this was

118   equal to the concentration found in the undiluted sample). The same chronoamperometric procedure was used, as

119   previously described.

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3. RESULTS AND DISCUSSION

3.1 Principles of the Amperometric Measurement of Fructose

$$\text{Fructose} + \text{FDH}_{Ox} \rightarrow \text{FDH}_{Red} + \text{5-dehydro-D-fructose} \tag{1}$$

$$\text{FDH}_{Red} + 2\text{Fe}^{3+} \rightarrow 2\text{Fe}^{2+} + \text{FDH}_{Ox} \tag{2}$$

$$2\text{Fe}^{2+} \rightarrow 2\text{Fe}^{3+} + 2e^{-} \tag{3}$$

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.

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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.

142   Fig.2 shows a comparison of the cyclic voltammetric behaviour of an unmodified screen printed carbon electrode (a)

143   and a SPCE with 17.89 µg deposited onto the surface (b). The increase in current magnitude may be explained by an

144   enhancement in the electron transfer properties, from ferrocyanide to the modified electrode surface. It should be

145   mentioned that the coefficient of variation was determined for the anodic peak currents; this was found to be

146   reduced from 6.73% to 4.75% (n=9), for plain SPCE and SPCE-G-COOH, respectively. For the development of the

147   chronoamperometric assay this improved precision, together with the improved sensitivity, can be considered of

148   importance to the development of a reliable analytical procedure.
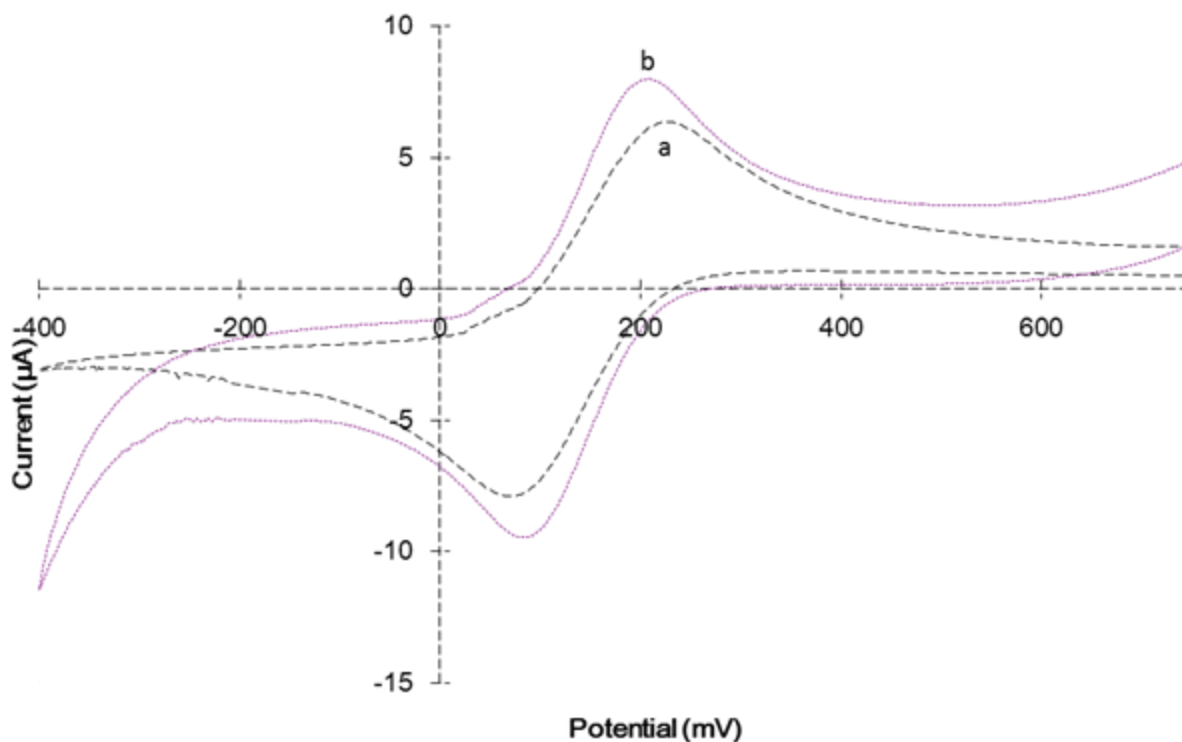
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150   *Fig.2. A typical cyclic voltammograms obtained with 0.5 mM ferricyanide in 0.1 M phosphate buffer pH 7.5*

151   *containing 0.1 M potassium chloride using a scan rate of 10 mV s^{-1}: for (A) plain SPCE (B) SPCE-G-COOH.*

152   *Voltammetric conditions: starting potential +0.8 V, switching potential -0.4 V.*

153

154   Fig.2 also shows that there is a difference in the peak separation (ΔE_p) for the two voltammetric scans, for the plain

155   SPCE and SPCE-G-COOH; these values were 169 mV and 148 mV, respectively. Both values indicate that the

7

156   redox reaction is quasi-reversible; however, the reaction at the SPCE-G-COOH appears to be more favourable.

157   Further evidence for this is obtained the charged transfer coefficient (α), using equation 4 (Kirsch, Hart, Bird,

158   Luxton and McCalley, 2001) where is n is the number of electrons involved in the rate determining step.

159 $$\alpha n = \frac{0.048}{E_p(V) - E_{\frac{p}{2}}(V)}$$ (4)

160   The α values (obtained using n=1) for the plain SPCE and SPCE-G-COOH were calculated to be 0.56 and 0.64,

161   respectively. Therefore the electron transfer kinetics are more favourable in the case of the SPCE-G-COOH, which

162   indicates that this sensor has superior electrochemical characteristics, which should lead to more reproducible

163   measurements. It should be mentioned that both of these values are better that reported for a commercial graphite

164   electrode (reported α = 0.486) (Botasini, Marti and Méndez, 2016). From these results, it appears that the reason for

165   the increase in current magnitude occurs as a result of improved electron transfer, rather than a simple increase in

166   electrode surface. Consequently, all further studies were performed using the SPCE-G-COOH electrodes.

167   **3.3 Calibration Studies, using chronoamperometry with a SPCE-G-COOH**

168   We began this study by investigating the SPCE-G-COOH modified with 1 U of FDH deposited onto the surface; a 3

169   minute incubation time was employed at room temperature, at open circuit. A calibration study was carried out over

170   the range 0.10 to 1.00 mM fructose; a linear response was obtained under these conditions and the slope was found

171   to be 10.085 µA mM^{-1}, Fig.3A(a).

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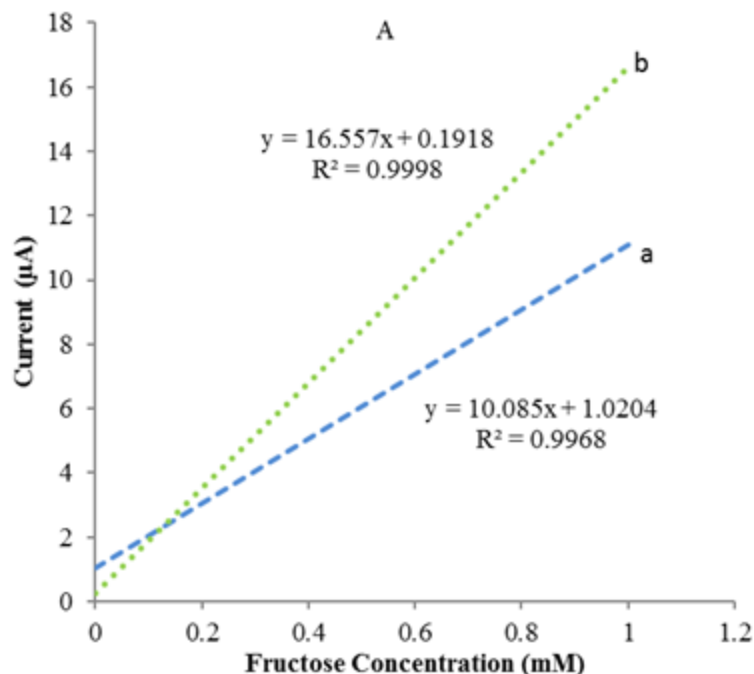
$y = 16.557x + 0.1918$
$R^2 = 0.9998$

$y = 10.085x + 1.0204$
$R^2 = 0.9968$

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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.

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 $\mu A\ mM^{-1} \pm 0.4\ \mu A\ mM^{-1}$.

Fig.3B shows typical chronoampergrams 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 $\mu A\ mM^{-1}\ cm^{-2}$, which

allows comparison with the current assay: Nicholas, et al. 2017 (current study) 58.56 $\mu A\ mM^{-1}\ cm^{-2}$; Biscay, Rama,

García, Reviejo, Carrazón, García, 2012 (ferrocyanide modified SPCE) 9.95 $\mu A\ mM^{-1}\ cm^{-2}$; Trivedi, et al. 2009

(amperometric biosensor using FDH) 2.19 $\mu A\ mM^{-1}\ cm^{-2}$; Antiochia, et al., 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.

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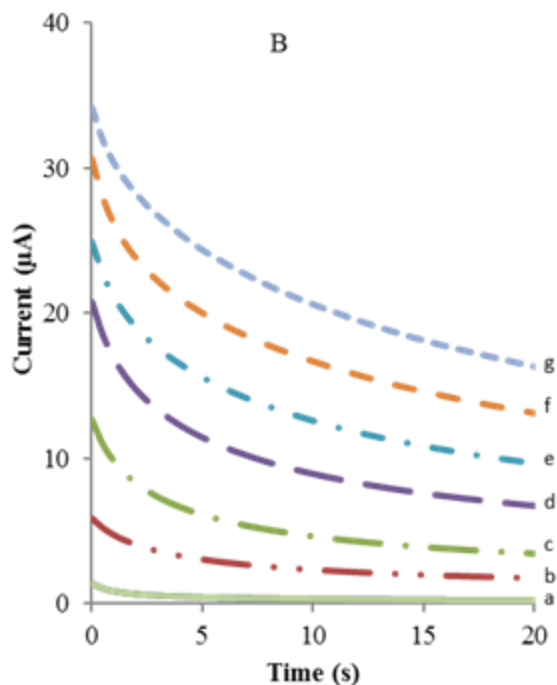
Fig. 3B Cchronoamperograms obtained with the fructose sensor for various concentrations of fructose: a) 0.00 mM;

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
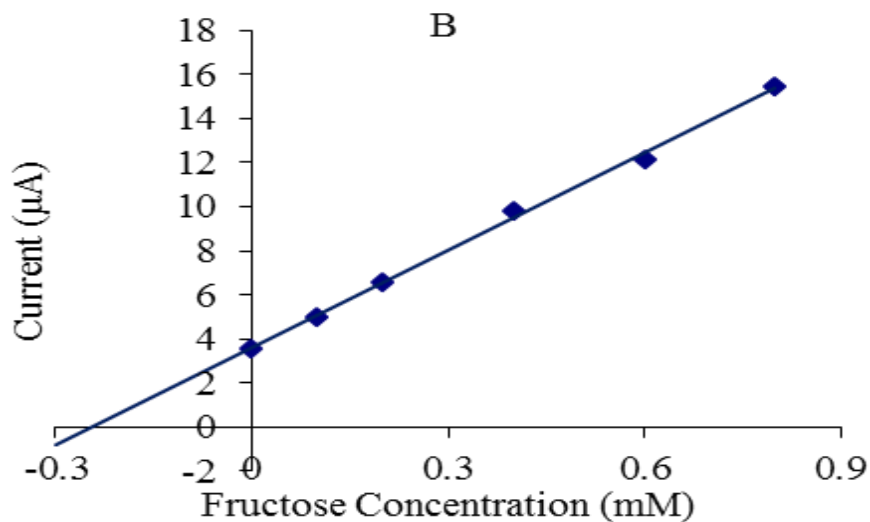
FDH with a 3 minute incubation time.

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.
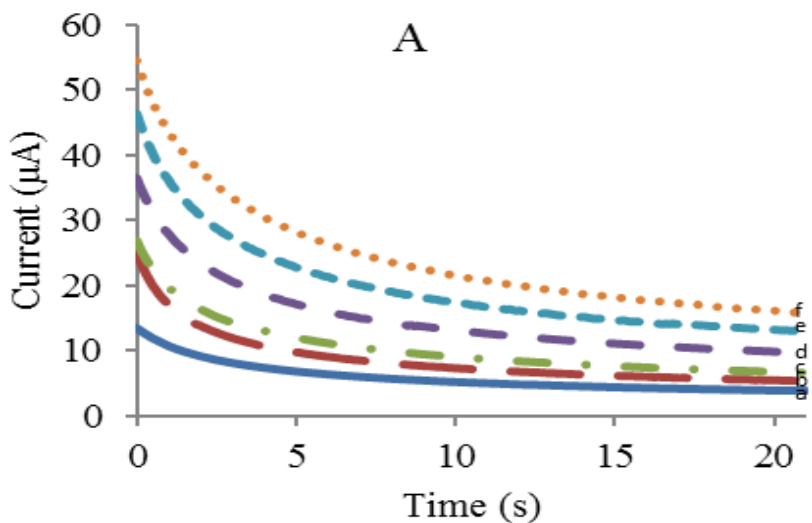
201

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.

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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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210

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

212 should be noted that column 2 refers to the concentration on the screen-printed transducer; column 3 refers to the

213 concentration of fructose corrected for the dilution factors.

214 **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

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216 The data in the Table 1 indicates that the mean original concentration of fructose was 477.2 mM; the precision data

217 of 3.031 % suggests that the method shows promise for analysis for fructose in fruit juices.

218 It is known that ascorbic acid is present in apple juice of concentrations around 2.15 mM (SELFNutrition Data,

219 2014). In order to determine whether this vitamin would affect the response obtained for fructose, in the apple juice

220 sample, a fixed concentration of 2.15 mM ascorbate was added to the neat apple juice followed by dilution (as

221 described earlier). The resulting chronoamperograms did not show any increase in anodic current for any of the

222 solutions shown in Table 1. This is perhaps not surprising bearing in mind the high ratio of fructose to ascorbic acid,

223 present in the sample. This study demonstrated that ascorbic acid at the levels present in the commercial apple juice

224 did not influence the magnitude of the fructose response; therefore no complicated sample preparation procedures

225 were required. The recovery of fructose, added to the original apple juice sample is summarised in Table 2.

226 **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 %

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228 The data in Table 2 shows that the mean recovery of added fructose is 97.12 % and the co-efficient of variation of

229 6.42 %. Clearly the data shown in Table 1 and Table 2 demonstrates that the chronoamperometric bioassay should

230 give reliable data for the analysis of fructose concentration in fruit juice products.

231

232   **4. Conclusion**

233 This paper describes the development of a simple, low cost chronoamperometric assay, based on the electrocatalytic

234 oxidation of FDH with a SPCE-G-COOH, and its evaluation using a commercial fruit juice. It was shown that the

235 incorporation of modified graphite nanoparticles enhanced the sensitivity and improved the precision of the response

236 towards the enzymatically generated ferrocyanide species, compared with a plain SPCE. The calibration studies

237 indicated that a linear response could be obtained up to 1.00 mM fructose using a mediator concentration of 3 mM.

238 Consequently, the proposed biosensor could be adapted for a wide range of food products. It should be mentioned

239 that sample preparation only involves the dilution of the sample prior to analysis; which is performed only on 40 µl,

240   of solution, applied directly onto the sensor surface. Therefore, it is readily feasible that this approach could be

241   performed near to the point of production and used as a quality control method. It should be mentioned that the

242   detection limit of 8 µM was achieved in the current study; however the detection limits in the commonly used Brix

243   test has a limit of detection of 556 µM (Cejpek 2012) and a method involving HPLC with UV-visible spectrometry

244   had reported a detection limit of 222 µM (Bevers HAJM, Wijntje R and de Haan AB 2005). Therefore, we believe

245   that the current amperometric fructose bioassay, employing a SPCE-G-COOH, holds promise for applications where

246   other conventional techniques do not have the desired detection limit.

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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.

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

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