

1 **Rapid sucrose monitoring in green coffee samples using multienzymatic biosensor**

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3 Miroslav Stredansky^{a,d}, Luca Redivo^{b*}, Peter Magdolen^c, Adam Stredansky^d, Luciano Navarini^e4 ^aDepartment of Analytical Chemistry, Faculty of Chemical and Food Technology, Slovak University of
5 Technology, Radlinskeho 9, 81237 Bratislava, Slovak Republic6 ^bDepartment of Chemistry and Biochemistry, School of Biological and Chemical Sciences, Queen Mary
7 University of London, Mile End Road, London, E1 4NS, United Kingdom8 ^c Institute of Chemistry, Slovak Academy of Sciences, Dubravská cesta 9, 84538 Bratislava Slovak Republic9 ^d Biorealis s.r.o., Radlinskeho 9, 81107 Bratislava, Slovak Republic10 ^e illycaffè S.p.A., Via Flavia 110, 34147 Trieste, Italy

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13 *E-mail: l.redivo@qmul.ac.uk

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15 **Abstract**

16 Amperometric biosensor utilizing FAD-dependent glucose dehydrogenase (FAD-GDH) for a specific
17 sucrose monitoring in green coffee is described. FAD-GDH was co-immobilized with invertase and mutarotase on
18 a thin-layer gold planar electrode using chitosan. The biosensor showed a wide linearity (from 10 to 1200 μM),
19 low detection limit (8.4 μM), fast response time (50 s), and appeared to be O₂ independent. In addition the
20 biosensors exhibited a good operational (3 days) and storage (1 year) stability. Finally, the results achieved from
21 the biosensor measurements of sucrose in 17 samples of green coffee (*Coffea arabica*, *C. canephora* and *C. liberica*)
22 were compared with those obtained by the standard HPLC method. The good correlation among results of real
23 samples, satisfactory analytical performance and simple use of the presented biosensor make it suitable for
24 application in coffee industry.

25

26 **Keywords:** Sucrose; Biosensor; Green coffee; Rapid analysis, Amperometry

27 **1. Introduction**

28 Coffee is the most commercialized food product and most widely consumed beverage in the world. In
29 2010, coffee production reached 8.1 million tons worldwide which represents more than 500 billion cups. The cup

30 quality is affected primarily by the composition of green coffee being influenced with agricultural practices,
31 environmental factors, variety and maturity (Farah, 2012). Sucrose is one of the major constituent of green coffee
32 and is responsible for coffee flavour and quality (Yigzaw Dessaleng et al., 2007). It is an important precursor of
33 taste and aroma developed during the roasting process. Besides Borém et al. (2016) recently found that the level
34 of sucrose is a good discriminant marker for the beverage quality. For instance, the higher sucrose content is one
35 of the reasons for the superior aroma and overall flavour of Arabica coffee in comparison to Robusta one. In fact,
36 Arabica contains from about 6 to 11 % and Robusta from 3 to 7 % of sucrose in green beans (Ky et al. 2001;
37 Campa et al., 2004; Knopp et al., 2006; Farah, 2012).

38 Several methods have been used for determination of sucrose in green coffee, including high performance
39 liquid chromatography (HPLC; O'Driscoll, 2014; Borém et al., 2016), anion-exchange chromatography coupled
40 to pulsed amperometric detection (Ky et al., 2001), enzymatic spectrophotometric method (Alcázar et al., 2005),
41 near infrared spectroscopy (Aluka et al., 2016; Santos et al., 2016). However, these methods require expensive
42 laboratory equipment and educated personnel. Moreover, the HPLC analyses are time-consuming. Biosensors can
43 represent an alternative method to overcome these drawbacks (Monošík et al. 2012a). They exhibit rapid response,
44 high selectivity, cost effectiveness, and they provide an option to perform analysis in situ due to their ability to be
45 miniaturized. Various enzymatic compositions and detection principles were described for the construction of
46 sucrose biosensors. Sole invertase (INV) was used for the thermometric (Thavarungkul et al., 1999) or fluorescent
47 (Bagal-Kestwal et al., 2015) biosensors. The combination of INV, glucose oxidase (GOX) and mutarotase (MUT)
48 was employed for conductometric (Soldatkin et al., 2013; Pyeshkova et al., 2015) and amperometric
49 (Surareungchai et al., 1999; Gouda et al., 2001; Majer-Baranyi et al., 2008). The simultaneous use of INV and
50 fructose dehydrogenase (FDH) was presented, too. However, FDH is relatively expensive enzyme and the GOX
51 based biosensors are susceptible to oxygen concentration in the measuring media, which can lead to a decrease in
52 the signal, and underestimation of measured values in cases where artificial mediators are used (Tang et al., 2001).
53 Recently we have proposed the implementation of FAD-dependent glucose dehydrogenase (FAD-GDH) in the
54 biosensor for glucose analyses in various beverages (Monošík et al., 2012b). This commercially available
55 convenient enzyme exhibits no dependency on oxygen and a high stability.

56 The aim of the present study was to develop a sucrose biosensor based on the combination of three
57 enzymes (INV, MUT, FAD-GDH) suitable for the rapid and selective sucrose analysis in green coffee and
58 compatible with the portable analytical device Omnilab currently serving beverage producers as an alternative to
59 classic analytical methods.

60

61 2. Experimental

62

63 2.1. Materials

64 Glucose dehydrogenase FAD-dependent (GDH-FAD, 1160 U mg⁻¹ solid) was purchased from Sekisui
65 Diagnostic (Tokyo, Japan), and is reported to have been isolated for *Aspergillus sp.*, invertase and mutarotase from
66 Sorachim (Lausanne, Switzerland). Meldola blue, Azure A, Azure C, methylene blue, thionine, N-
67 methylphenazonium methyl sulfate, sucrose, trehalose and chitosan from shrimp shells (85% deacetylated) were
68 supplied by Sigma-Aldrich (St. Louis, USA). Potassium phosphate monobasic and potassium phosphate dibasic
69 were purchased from Riedel-de Haen (Seelze, Germany). Water deionized by a Millipore Milli-Q purification
70 system was used. All chemicals used were of analytical grade. Gold planar electrodes with diameter of 1.6 mm
71 equipped with Ag/AgCl reference electrode (diameter 2 mm, screen-printed) deposited on the planar glass-epoxy-
72 laminate substrate were obtained from Biorealis (Bratislava, Slovakia).

73 Nine different samples of green *Coffea arabica* L. beans (geographical origin: El Salvador, India,
74 Ethiopia, Brazil, Indonesia, Tanzania, Colombia), five different samples of green *C. canephora* Pierre ex Froehner
75 var. robusta beans (geographical origin: Indonesia, Ivory Coast, Vietnam, Tanzania, Cameroon) and three different
76 samples of green *C. liberica* Bull ex Hiern beans (geographical origin: Indonesia) from commercial lots were used.
77 *C. arabica* sample from El Salvador was a Low Caffeine Bourbon (BLC) cultivar.

78

79 2.2. Apparatus

80 Electrochemical measurements were performed with electrochemical analyzers Autolab M101 (Metrohm
81 Autolab, Netherlands) and Omnilab from Biorealis (Bratislava, Slovakia)

82 Reference HPLC assays were run on Waters 600E HPLC System (Waters, Milford, USA) equipped with
83 the refractometer detector (model PU 4026, Philips, Eindhoven, Netherlands).

84

85 2.3. Preparation of biosensors

86 The planar gold electrodes were cleaned with Milli-Q water and ethanol. The immobilization of the
87 enzymes on the electrode surface was carried out by their sandwiching between (1 % w/w) chitosan layers. Each
88 layer was deposited after the previous one was dried. All enzymes were dissolved in Milli-Q water before

89 procedure. The prepared biosensors were stored at room temperature in a desiccator until use. The details on the
90 quantities of enzymes are given in Results and Discussion.

91

92 *2.4. Preparation of green coffee samples*

93 Green coffee beans were ground to a fine powder using a mixer mill Retsch MM400 (Retsch GmbH,
94 Germany). Then 2 g of each sample were deposited into a 100-mL flask, mixed with 40 ml of deionized water,
95 heated up to the boiling point agitated and left slowly until laboratory temperature. The extracts were subsequently
96 filtered through a fine paper.

97

98 *2.5. Amperometric measurements*

99 Electrochemical measurements were performed with electrochemical analyzers Autolab M101 (Methrom
100 Autolab, Netherlands) and Omnilab from Biorealis (Bratislava, Slovakia). Chronoamperometry was performed by
101 applying selected constant potential (vs. Ag/AgCl) after inserting the biosensor in volume of a measuring solution
102 either 1 mL in microtube or 10 mL in beaker under stirring at laboratory temperature. Values from -300 mV to
103 +300 mV were tested for the optimization of working potential. The pH values of a 0.1 M phosphate buffer solution
104 (PBS) were optimized from pH 5.0 to 8.0. Similarly, the suitable concentrations of electrochemical mediators
105 (from 0.1 to 2 mM) in the working media were also investigated. The biosensors were stored after measurements
106 in 0.1 M PBS of pH 6.0 at laboratory temperature (up to 10 hours) or at 4 °C (for longer operational stability
107 studies). The biosensors were kept dry in a desiccator at laboratory temperature for the storage stability studies.

108

109 *2.6. HPLC analysis*

110 Reference HPLC assays of sucrose were run on Waters 600E HPLC System (Waters, Milford, USA)
111 equipped with the refractometer detector (model PU 4026, Philips, Eindhoven, Netherlands). The analytical
112 conditions were as follows: column Polymer IEX in H⁺ form 250 mm x 8 mm, 8µm in diameter (Watrex,
113 Bratislava, Slovakia); column temperature 80 °C and pressure 300 Psi; mobile phase Milli-Q water; flow rate 1.0
114 mL min⁻¹. Data were collected and processed by Clarity chromatography station DataApex (Prague, Czech
115 Republic). Samples were diluted in a mobile phase and filtered through 0.22 µm Chromafil AO filters, Macherey-
116 Nagel (Düren, Germany) prior to analysis. Sugars were identified by comparison with retention times and co-
117 elution of authentic standard solutions.

118

119 3. Results and discussion

120 The principle of the presented biosensor is illustrated in Figure 1. It is based on the amperometric detection
121 of reduced electron acceptor, further referred to as mediator (Med), which is generated during the course of the
122 GDH-FAD-catalyzed oxidation of β -D-glucose formed from sucrose by the co-immobilized INV and MUT. The
123 GDH-FAD enzyme was previously employed in the development of glucose specific biosensor and its specificity
124 is reported in the work by (Monošík R. *et al.*, 2012b). From this study, the high specificity for β -D-glucose of
125 GDH-FAD enzyme was proved against other sugars, alcohols, and acids. The reduced mediator is oxidized on the
126 electrode surface and the resulting current proportional to the analyte concentration is measured. Gülce et al. (1995)
127 reported that phosphate ions used in the medium at a high concentration catalyse the conversion of α -glucose to
128 β -glucose, eliminating the need for MUT. When we applied the high level of phosphates instead of MUT the
129 biosensor response became sluggish. Another possible principal problem of the used enzyme cascade comes from
130 the fact that glucose presented in real samples could cause an interference, but its content in green coffee is
131 negligible in comparison with sucrose (Knopp et al., 2006; Smrke et al., 2015). Besides small amounts of glucose
132 in coffee samples did not influence the results obtained by the sucrose biosensor because differential measurements
133 were applied and the signal obtained by the biosensor without invertase (measuring only glucose) was subtracted
134 from the signal of the sucrose biosensor (measuring sucrose + glucose).

135

136 3.1. Optimization of biocatalytic layer

137 The quantities of enzymes on the electrode surfaces were optimized from 0.5 to 15 U. The optimal
138 amounts of 6.0 U of FAD-GDH, 1.75 U of MUT, and 2.5 U of INV were found for immobilization on the electrode.
139 Higher enzyme loadings induce the significant current decrease, which is probably caused by a partial blocking of
140 the electrode surface with the large mass of protein. By contrary, lower enzyme quantities led to the decline of
141 biosensor sensitivities and narrow linear ranges. The enzymes were immobilized on the electrode surface by their
142 sandwiching between chitosan layers. Similarly, to our previous works (Monošík et al., 2012b; Monošík et al.,
143 2013), the chitosan concentration of 1 % (w/w) showed the best results. Finally, the addition of 1.5% of trehalose
144 in the solution of enzymes before their spreading on the electrodes improved the sensibility and stability of the
145 biosensors. The use of trehalose is a common practice to improve the long-term stability and activity of enzymes,
146 especially in the dried state, which is the condition for the storage of the biosensor. The long-term storage stability
147 results in an improved enzyme functionality and therefore sensor sensitivity. The mechanism of action of trehalose
148 is explained in more details in other works (Kaushik & Bhat, 2003; Olsson, Jansson, & Swenson, 2016).

149

150 *3.2. Optimization of working conditions*

151 The pH of working media is a very important factor affecting the biosensor performance, particularly in
152 the case of multienzymatic biosensors. The pH dependence of the presented biosensor was investigated over the
153 range from 5.0 to 8.0 in 0.1 M PBS. The highest relative response was obtained at pH 5.75 which corresponds to
154 the optimum of FAD-GDH (Monošík et al. 2012b) and it is the compromise between the optimum values of INV
155 (3.5-4.0) and MUT (7.4) given by their supplier. The concentration of the PBS showed a low effect in the range
156 from 0.025 to 2.0 M, and next experiments were performed in 0.1 M PBS.

157 The selection of a good electrochemical mediator is important for the good functionality, sensitivity and
158 selectivity of the amperometric biosensors. The suitable mediator accelerates an electron transport from the
159 enzyme to the electrode surface and determines the working potential. The possibility to apply low potential allows
160 a substantial reduction of eventual interferences coming from electroactive compounds presented in real samples,
161 such as polyphenols, ascorbate, etc. Green coffee contains a very high quantity of polyphenols (various chlorogenic
162 acids), up to 12% of its dry weight (Farah, 2012). Chlorogenic acids showed oxidation peaks about +225 mV
163 against Ag/AgCl reference electrode (Šeruga & Tomac, 2014). It means lower working potentials should be used
164 to eliminate this interfering current of the oxidation of chlorogenic acids during sucrose measurements with
165 biosensors. The use of mediators from groups of phenothiazine or phenoxazine dyes allows working at low
166 potentials. Monošík et al. (2012b) utilized N-methylphenazonium methyl sulfate at +50 mV for the glucose
167 biosensor based on FAD-GDH. Here we tested the following dyes: N-methylphenazonium methyl sulfate, Meldola
168 blue, Azure A, Azure C, methylene blue and thionine. All of them showed the highest biosensoric responses
169 between -200 and +50 mV (vs. Ag/AgCl). The best results derived from the use of 0.5 mM Azure C at -100 mV,
170 which we chose for next study. No interferences from green coffee extracts were observed at these conditions
171 using the bare electrode without enzymes. Therefore, this potential permits satisfactory sucrose measurement
172 sensitivities and simultaneously avoids undesirable interferences.

173

174 *3.3. Analytical performance*

175 The analytical studies were performed at the optimal working conditions in 1 mL of PBS in microtube
176 under stirring (at laboratory temperature) by additions of 10 mM sucrose solution. The resulting calibration plot
177 (Figure 2) was linear over the range from 10 to 1200 μ M with a correlation coefficients $R^2 = 0.998$ ($n=11$) (the
178 equation is reported below).

179
$$y = 7.030 (\pm 4.772) + 0.647 (\pm 0.008) \times \text{Sucrose concentration } (\mu\text{M})$$

180 The biosensor showed a detection limit of 8.4 μM with the sensitivity of 0.65 nA μM^{-1} . Limit of detection is based
181 on signal/noise = 5. The time required to reach steady-state response was 50s. These results are comparable to
182 those obtained with the amperometric biosensors reported previously (Surareungchai et al., 1999; Gouda et al.,
183 2001; Majer-Baranyi et al., 2008; Vargas et al., 2013; Antiochia et al., 2014).

184

185 3.4. Reproducibility and stability

186 The reproducibility of the biosensor measurements was carried-out by consecutive addition of 10 μL of
187 standard sucrose solution (10mM) in 1 mL PBS solution. The average response of the biosensor was 71.3 ± 1.8
188 nA (n=10, R.S.D.= 2.28%). This finding confirms the reliability of the biosensor for analysis of real samples.

189 Long-term storage stability of biosensors is one of the most important parameters in case of their potential
190 commercial use. Humidity and high temperatures are the most negative factors, which can affect the storage
191 stability of enzymatic biosensors. The presented biosensors held in a desiccator at room temperature without use,
192 kept more than 90% of the initial response ability at least after 12 months. Moreover, they were resistant against
193 50 °C heat for at least 5 days, which proves the stability of the sensor for shipment also in summer. The stability
194 monitoring yet continues. Among the described sucrose biosensors only the one reported by Antiochia et al. (2014)
195 showed the comparable stability retaining 80% of the original response after 4 months.

196 Operational stability is also required for the evaluation of biosensor performance because describes the
197 stability of the biosensor during routine analysis. To assess the capability of the sucrose biosensor for routine
198 analysis standard sucrose solutions were measured in various intervals to simulate a real use. Between
199 measurements, biosensors were stored in PBS at laboratory temperature and overnight at 4°C. The biosensors did
200 not show any loss of activity after 60 analyses in a row and after 24 hours of use. All of them exhibited response
201 ability above 75% after 4 days. Some sucrose biosensors based on combination of INV and GOX (Gülce et al.,
202 1995; Surareungchai et al., 1999) or FDH (Vargas et al., 2013; Antiochia et al., 2014) showed comparable or better
203 operational stabilities. But these biosensors are constructed using classical disc electrodes (Pt, Au, carbon paste)
204 which are not convenient for a low-cost mass production. On the other hand, the here presented biosensor, have a
205 simple concept, and is based on commercially available cheap planar electrodes, which are easily processable, and
206 the enzyme and chitosan layers could be deposited onto the planar substrate by well-known printing techniques.
207 Thus, a more frequent sensor exchange is acceptable.

208

209 3.5. Sucrose analysis in green coffee

210 Although the biosensor showed good analytical performance when using pure sucrose solutions, it was
211 necessary to assess the performance of the biosensor with respect to more complex real samples and to compare
212 the results with those obtained by a standard analytical method. It is an important step for a verification of
213 biosensor's accurateness to measure real samples. Considering the linear range of the biosensor and the sugar
214 levels in green coffee, the extraction by the 20-fold amount of water allowed direct biosensoric analyses without
215 further dilution and any other pre-treatment. The sucrose determination was performed by successive injecting 10
216 μL of sample and calibration solution in 1 mL of PBS of pH 5.75 containing 0.5 mM Azure C. The measurements
217 of 17 green coffee (*C. arabica*, *C. canephora* and *C. liberica*) samples were performed simultaneously with the
218 standard HPLC method (Table 1). A satisfactory correlation was obtained between the biosensor and the HPLC
219 techniques results.

220 Figure 3 compares the performance of the proposed biosensor against the HPLC method. The obtained correlation
221 equation and its linearity are reported below.

$$222 \quad y = 0.059(\pm 0.311) + 0.995(\pm 0.047)x \quad R^2 = 0.965$$

223 The correlation between the two set of data is good, as evident from the slope of the fitted line very close to 1 and
224 the low intercept value. These data confirm the validity of the proposed biosensor for accurate and reliable sucrose
225 analysis in green coffee beans.

226 As expected, no interferences coming from green coffee constituents were observed at the selected measuring
227 conditions. This opens the possibility to adopt the rapid, easy and convenient application of the presented sucrose
228 biosensor by coffee industry. The content of fructose and glucose measured by HPLC in the used samples was
229 negligible. Only some African green coffee samples (Ethiopia, Cameroon, Tanzania, and Ivory Coast) contained
230 slightly higher amounts of glucose (from 0.23 to 0.42 %) and fructose (from 0.24 to 0.95 %). These data were
231 confirmed also by measuring with the glucose and fructose specific biosensors, described previously by our group
232 (Monošík et al., 2012b; 2013).

233

234 4. Conclusions

235 A novel multienzymatic biosensor selectively quantifying sucrose in green coffee based on commercially
236 available materials is reported. INV, MUT and FAD-GDH were co-immobilized between chitosan layers on the
237 surface of thin-layer planar gold electrodes. The simple and effective immobilization technique provided long-
238 term storage stability, low fabrication costs, and good analytical performance. The biosensor exhibited a wide

239 linear range (10-1200 μM), low detection limit (8.4 μM), high sensitivity (0.65 nA μM^{-1}), short measuring time
240 (50 s) and interference-free measurements. It was successfully applied to sucrose analysis in green coffee samples,
241 and validated through comparison with the reference HPLC method. Performance characteristics of this useful
242 analytical tool make it appropriate for coffee industry, as valid alternative of standard analytical techniques. The
243 developed biosensor is fully compatible with the small commercial biosensoric devices Omnilab and is now
244 commercially available.

245

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251

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324 chlorogenic acid, sucrose and trigolline contents among Ethiopian arabica coffee accessions. *Ethiopian*
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326

Figure Captions

327 Figure 1: Sucrose bioelectrode reaction scheme.

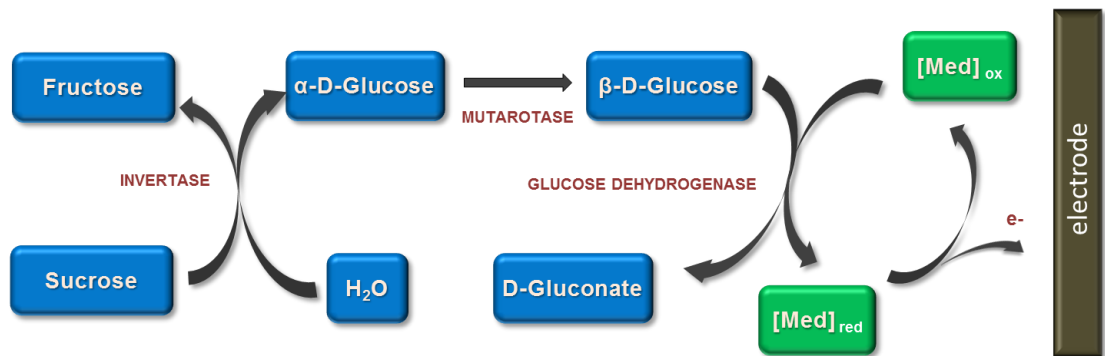
328 Figure 2: Calibration curve obtained for the sucrose biosensor. Experimental conditions: 0.5 mM Azure C, 0.1 M
329 phosphate buffer, pH 5.75, applied potential -100 mV vs. Ag/AgCl.

330 Figure 3: Graphical comparison between the sucrose determination in 17 green coffee sample, performed using
331 the proposed electrochemical biosensor and the comparative HPLC methodology.

332

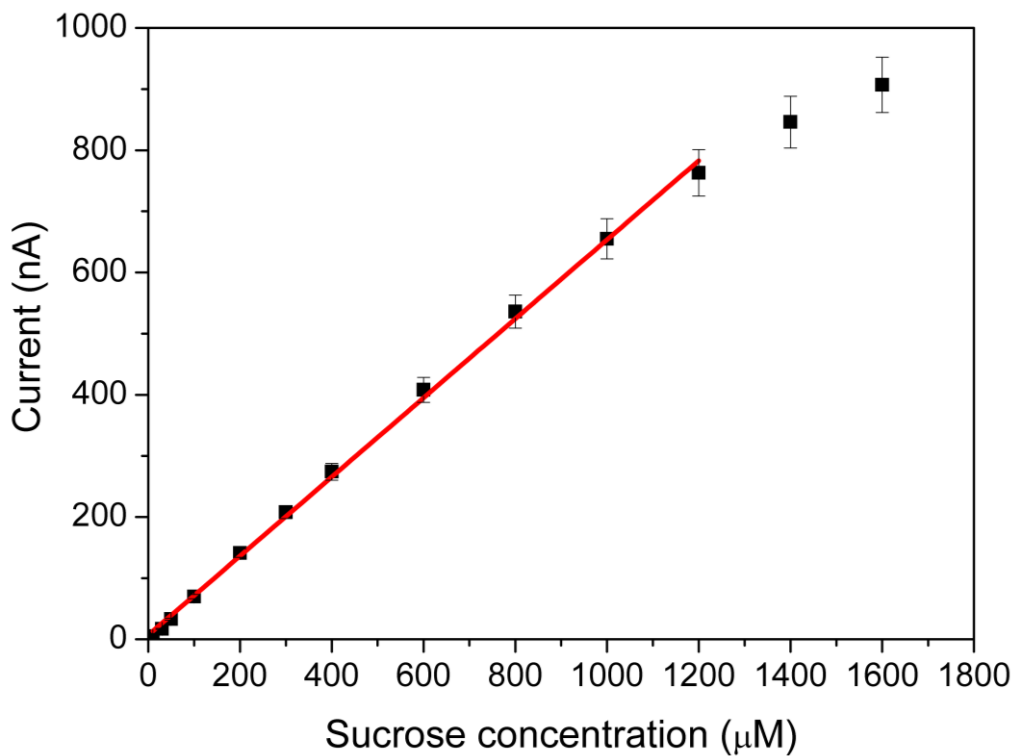
333

334 Figure 1



340 Figure 2

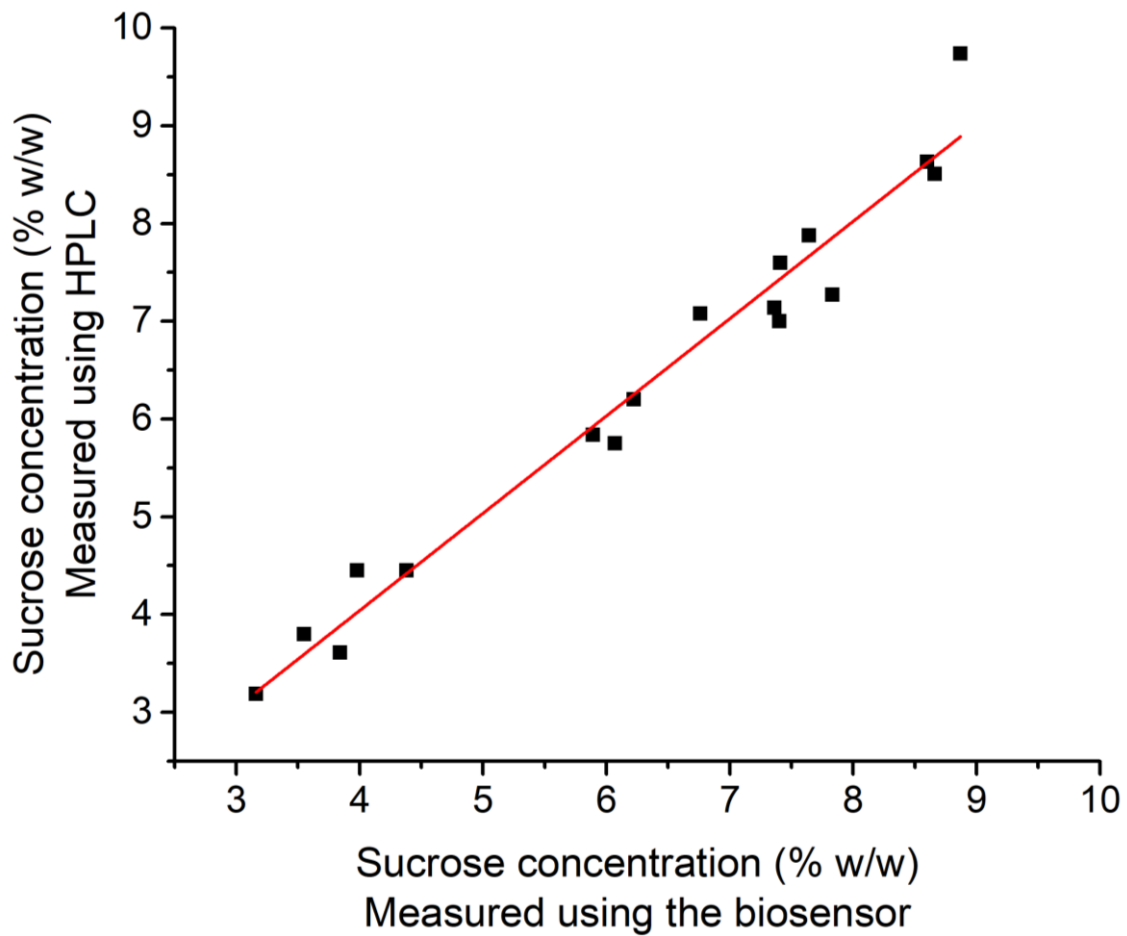
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343 Figure 3

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346 Table 1 Sucrose content analysis in 17 different green coffee beans using the developed biosensor and a
347 comparative HPLC method.

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Green coffee sample	Sucrose by biosensor (% w/w)	Sucrose by HPLC (% w/w)
Arabica BLC	8.66 ± 0.22	8.51 ± 0.30
Arabica India	7.36 ± 0.18	7.14 ± 0.25
Arabica Ethiopia	8.60 ± 0.31	8.63 ± 0.31
Arabica Brazil 1	8.87 ± 0.78	9.74 ± 0.35
Arabica Brazil 2	6.07 ± 0.18	5.75 ± 0.21
Arabica Brazil 3	7.41 ± 0.80	7.60 ± 0.27
Arabica Indonesia	7.83 ± 0.38	7.27 ± 0.25
Arabica Tanzania	7.64 ± 0.23	7.88 ± 0.28
Arabica Colombia	7.40 ± 0.62	7.00 ± 0.24
Robusta Indonesia	3.98 ± 0.26	4.45 ± 0.18
Robusta Ivory Cost	3.84 ± 0.16	3.61 ± 0.15
Robusta Vietnam	3.16 ± 0.14	3.19 ± 0.13
Robusta Tanzania	4.38 ± 0.19	4.45 ± 0.19

Robusta Cameroon	3.55 ± 0.21	3.80 ± 0.15
Liberica Indonesia 1	5.89 ± 0.44	5.84 ± 0.22
Liberica Asia	6.76 ± 0.25	7.08 ± 0.25
Liberica Indonesia 2	6.22 ± 0.12	6.20 ± 0.23

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