Rapid sucrose monitoring in green coffee samples using multienzymatic biosensor

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Abstract

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

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

1. Introduction

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

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

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

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

2. Experimental

2.1. Materials

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

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

2.2. Apparatus

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

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

2.3. Preparation of biosensors

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

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

2.4. Preparation of green coffee samples

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

2.5. Amperometric measurements

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

2.6. HPLC analysis

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

3. Results and discussion

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

3.1. Optimization of biocatalytic layer

The quantities of enzymes on the electrode surfaces were optimized from 0.5 to 15 U. The optimal 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. Higher enzyme loadings induce the significant current decrease, which is probably caused by a partial blocking of the electrode surface with the large mass of protein. By contrary, lower enzyme quantities led to the decline of biosensor sensitivities and narrow linear ranges. The enzymes were immobilized on the electrode surface by their sandwiching between chitosan layers. Similarly, to our previous works (Monošík et al., 2012b; Monošík et al., 2013), the chitosan concentration of 1 % (w/w) showed the best results. Finally, the addition of 1.5% of trehalose in the solution of enzymes before their spreading on the electrodes improved the sensibility and stability of the biosensors. The use of trehalose is a common practice to improve the long-term stability and activity of enzymes, especially in the dried state, which is the condition for the storage of the biosensor. The long-term storage stability results in an improved enzyme functionality and therefore sensor sensitivity. The mechanism of action of trehalose 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 μM with a correlation coefficients R^2 = 0.998 (n=11) (the
178 equation is reported below).

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

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

3.4. Reproducibility and stability

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

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

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

3.5. Sucrose analysis in green coffee

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

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

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

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

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

4. Conclusions

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

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

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Figure Captions

Figure 1: Sucrose bioelectrode reaction scheme.

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

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

Figure 1

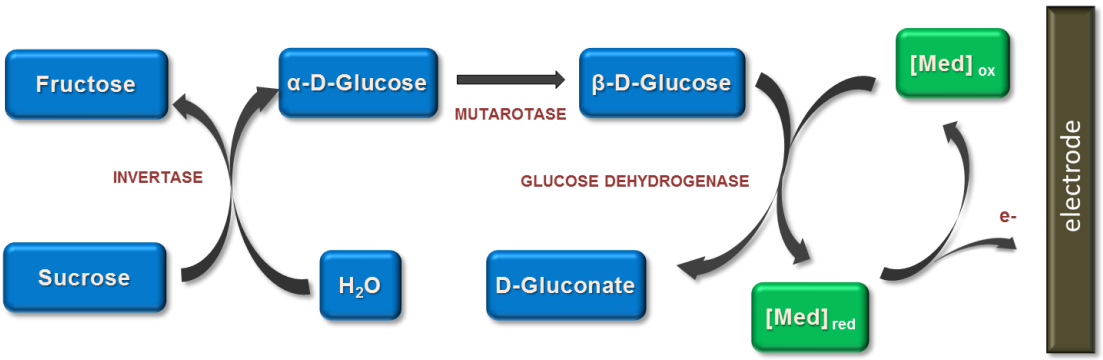
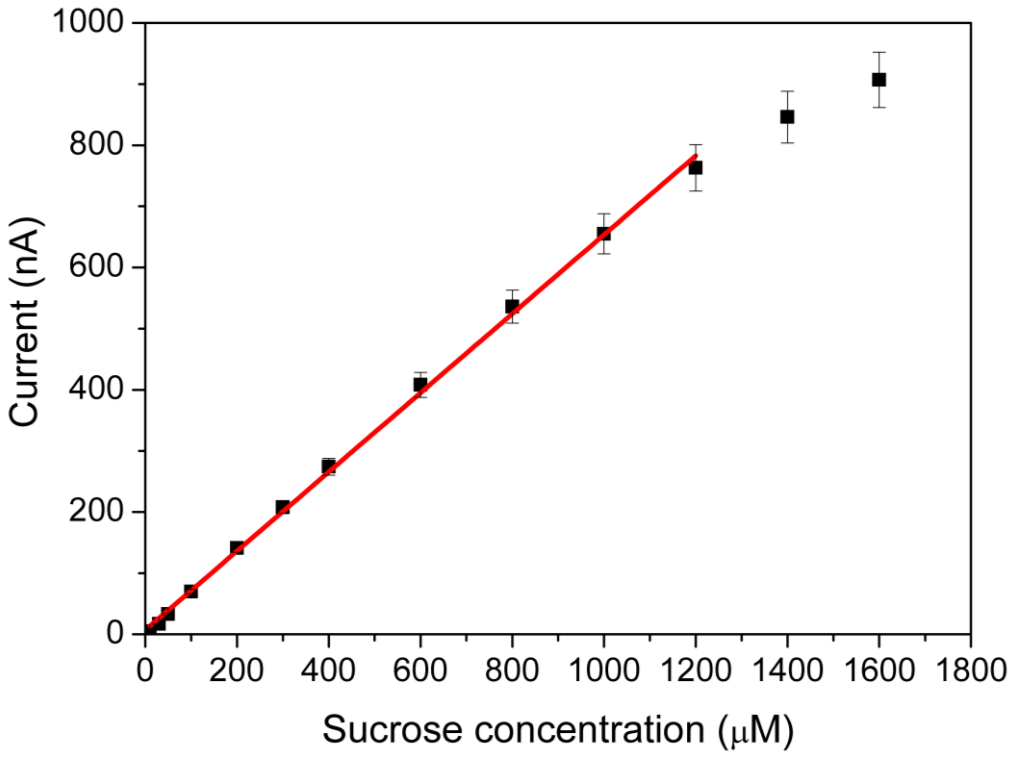


Figure 2



343 Figure 3

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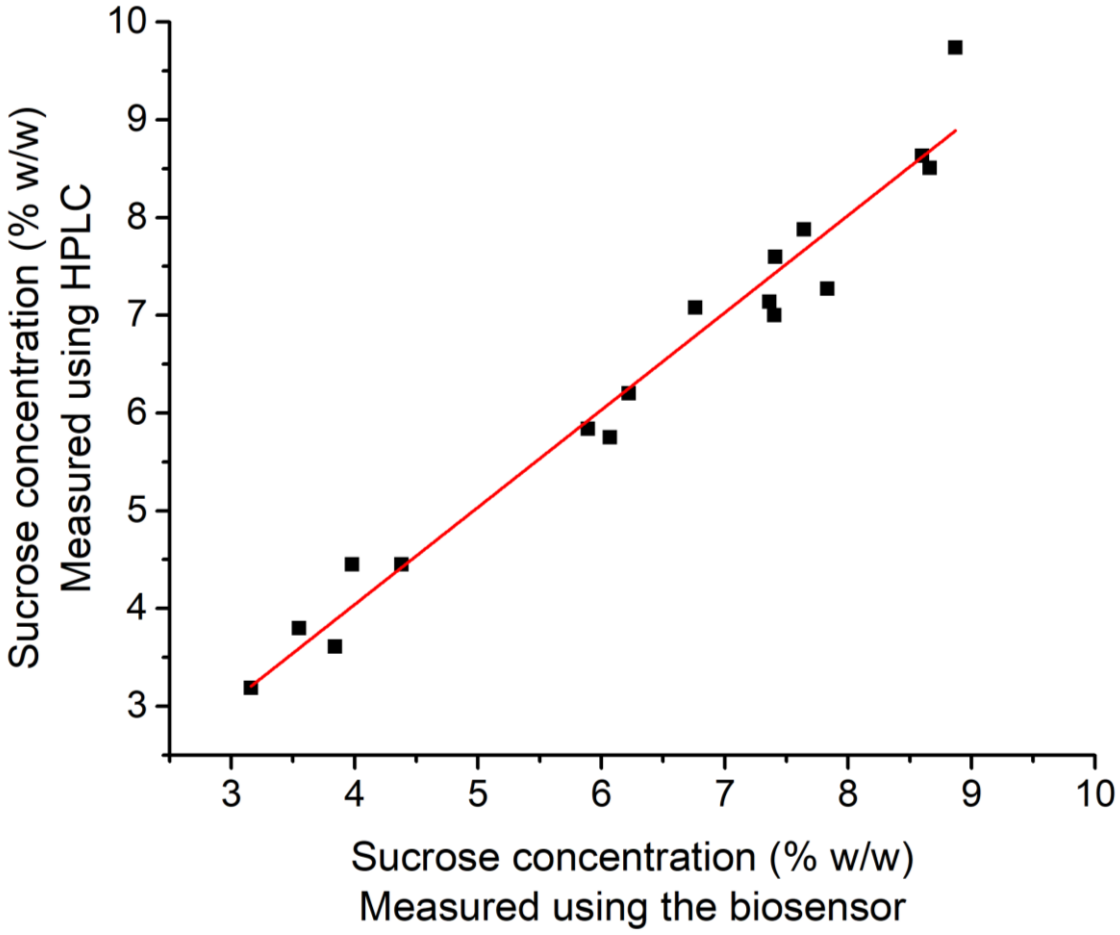

345

346 Table 1 Sucrose content analysis in 17 different green coffee beans using the developed biosensor and a

347 comparative HPLC method.

348

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