A Simple Method to Determine Bioethanol Production from Coffee Mucilage, Verified by HPLC

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This paper proposes a method to determine bioethanol concentration that uses a pycnometer verified with a high performance liquid chromatography (HPLC) technique; it is a simple tool to determine the density of liquids for getting information about the ethanol concentration. The results showed that the sugar concentration affected the bioethanol concentration. A lower initial sugar concentration of 26.5 g/L generated higher yield of 45.3% sugar to bioethanol and a fractional or relative yield of 88.74%. Significance tests were used to compare the two experimental means, revealing that the pycnometer method and HPLC provide the same bioethanol concentration with joint variances of 2.269, 0.242, and 0.112 for 3 different tests with initial sugar concentrations of 26.486 g/L, 49.043 g/L, and 68.535 g/L, respectively. This study established and developed a methodology to determine bioethanol concentration from coffee mucilage by the proposed method.

Keywords: Pycnometer; HPLC; Fermentation; Bioethanol concentration

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INTRODUCTION

Using renewable resources derived from biomass is beneficial when trying to develop sustainable energy systems from different substrates (Cortright *et al.* 2002). The conversion of biomass into biofuels can reduce the strategic vulnerability of petroleum-based transportation systems. Bioethanol has received considerable attention over the last few years as a fuel extender or even as a neat liquid fuel (Vučurović and Razmovski 2012). Previous studies reference the determination of ethanol or bioethanol content by HPLC (Liebmann *et al.* 2009) from polysaccharides (Huang *et al.* 2013), from steam-flaked sorghum and maize (Chuck-Hernandez *et al*. 2009), rapeseed straw (Karagöz *et al.* 2013), forestry residue (Ferreira *et al.* 2010), and others. Coffee mucilage is a waste product that is thrown directly into waterways without treatment. This is because it has no alternative use, which causes serious pollution problems.

Ethanol determination can be realized in several ways: densimetric methods (Lachenmeier *et al.* 2010), potassium dichromate (Breisha 2010), biosensor potentiometry (Rotariu *et al.* 2004), gas chromatography (GC) (Wang *et al.* 2003), capillary electrophoresis (Oliver *et al.* 2014), high performance liquid chromatography (HPLC) (Huang *et al.* 2013), Raman spectrometry (Shih and Smith 2009), near-infrared spectroscopy (NIR) (Liemann *et al.* 2009), beer analyzer, and flow injection analysis (Rangel and Tóth 2000).

For the dichromate oxidation spectrophotometry, the reagents used are highly toxic and harmful. Low stability, reproducibility, and accuracy are the disadvantages for enzymatic method, biosensor, and potentiometry. Raman spectrometry, capillary electrophoresis, GC method, and HPLC require expensive equipment. The HPLC method has a comparatively low sensitivity. NIR spectroscopy and analyzer have low accuracy.

A method proposed to determine bioethanol concentration in this paper using a pycnometer method verified with the high-performance liquid chromatography (HPLC) technique is like a simple tool to determine the density of liquids as a means for getting information about ethanol concentration. This method helps to compare the densities of two liquids, weighing the pycnometer with each liquid separately and comparing their masses (Pratten 1981; Piccolo and Bezzo 2009). The entire process should be conducted at a constant temperature to avoid errors due to slight variations in volume due to the temperature. The pycnometer is very sensitive to changes in concentration of salts in the water, so it is used to determine the salinity of the water and the density of biological liquids in laboratories, among other applications. The pycnometer method is an alternative way to quantify ethanol concentration in a fermentation medium when there is a lack of sophisticated equipment.

EXPERIMENTAL

Materials

Substrate

The coffee mucilage was extracted mechanically and supplemented with salts: 0.02 g/L of magnesium sulfate ($MgSO_4 7H_2O$) and 0.2 g/L of ammonium sulphate ($(NH_4)_2SO_4$) (Mishima *et al.* 2008).

Microorganisms

The yeast *Saccharomyces cerevisiae* Y-2034 (Wang *et al.* 2012) was maintained in a YPD medium (yeast extract 1%, peptone 2%, and dextrose 2%). The cells were incubated to 30 °C over 48 h (Mishima *et al.* 2008; Mussato *et al.* 2011). The strain was cultured in flasks of 250 mL under anaerobic conditions and stirred at 150 rpm at 30 °C over 24 h to allow cell growth to the exponential phase, and the cells were suspended in the fermentation medium.

Methods

Fermentation process

The fermentation process was carried out with three different reducing sugar concentrations of 26.486 g/L, 49.043 g/L, and 68.535 g/L, according to one factor design (Design Expert® Software Version 7.0.0, Stat-Ease, Inc. Minneapolis), by triplicate in a flask of 250 mL, pH 5, and temperature of 30 ºC (Reddy and Reddy 2011) with constant stirring at 150 rpm over 16 h. The volume used for the fermentations was 150 mL, with an initial cell concentration of 1.0×10^6 ufc/mL (Pratten 1981). Culture samples of 1 mL were taken at the end of the fermentation process and centrifuged at 10,000 rpm for 10 min at 5 ºC. The supernatant was filtered through a 0.22 m filter (Millipore, Bedford, MA, USA). After it was filtered, it was stored at -20 ºC for later analysis by HPLC.

Analytic methods

During the fermentation, the reducing sugar concentration was determined by the Miller method (Miller 1959). Viable cell counts were measured with a Neubauer chamber adapted with optical microscopy (Pereira *et al*. 2010), and trypan blue dye was used on the vital cells (Tolnai 1975). Bioethanol concentration was determined with a pycnometer method through densities verified with HPLC, using a column (©Phenomenex, Inc. USA) eluted at 60 °C with 0.0025 M H_2SO_4 at a flow rate of 0.5 mL/min and having a refractive-index detector.

Pycnometer method

A clean dry pycnometer was used for the next step with a capacity of 50 mL and a constant weight for each of the measurements. To prepare the medium used as a blank, it was necessary to obtain a flask fermented with the features described above (fermentation process). After the fermentation process, the alcohol formed in the fermentation medium from coffee mucilage was evaporated at 78 °C, cooled to 20 °C, and centrifuged at 10,000 rpm for 15 min at 5 °C. The supernatant was used as blank. For the calibration curve (Fig. 1), ethyl alcohol (99.9%) was added in the blank to have concentrations of ethanol from 0 to 50 g/L; the weights and densities determined by an analytical balance and a pycnometer were calculated at 20 °C.

To determine the concentration of ethanol in the fermentation, a sample of medium was taken, and centrifuged at 10,000 rpm for 15 min at 5 °C. The supernatant was changed to a new tube, and the precipitate was discarded. Each of the densities was determined at 20 °C, and the ethanol concentration was calculated from the calibration curve (AOAC 1990).

Density was calculated using the following equation:

$$\rho = \frac{m_1 - m_0}{v} \quad (1)$$

where is a density, m_1 pycnometer + sample mass, m_0 is pycnometer mass, and v is pycnometer volume.

Comparison of two experimental means

The results from a new analytical method can be contrasted by comparison with those obtained using a second method. In this case we have two sample means. The null hypothesis defines that the two methods provide the same result. In other words H_0: $\mu_1 = \mu_2$. It is necessary to test whether (y_1 - y_2) values differ significantly from zero. If the two samples have standard deviations that are not significantly different, then a joint estimation of the individual standard deviations s_1 and s_2 is carried out (Miller and Miller 2002).

For one to decide if the differences between two samples means, $\overline{y_1}$ y $\overline{y_2}$ is significantly different *i.e.*, for it to contrast the null hypothesis, H_0: $\mu_1 = \mu_2$, one has to calculate the *t* statistic:

$$t = \frac{(\bar{y}_1 - \bar{y}_2)}{s\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \quad (2)$$

where *n* is remarks and *s* calculated from:

$$s^2 = \frac{(n_1-1)s_1^2+(n_2-1)s_2^2}{n_1+n_2-2} \quad (3)$$

And t has $n_1 + n_2 - 2$ degrees of freedom.

RESULTS AND DISCUSSION

Determination of Bioethanol Concentration

In Fig. 1 the curve calibration is given with a coefficient of determination of 0.997. This was used to determine the bioethanol concentration in the fermentation step.

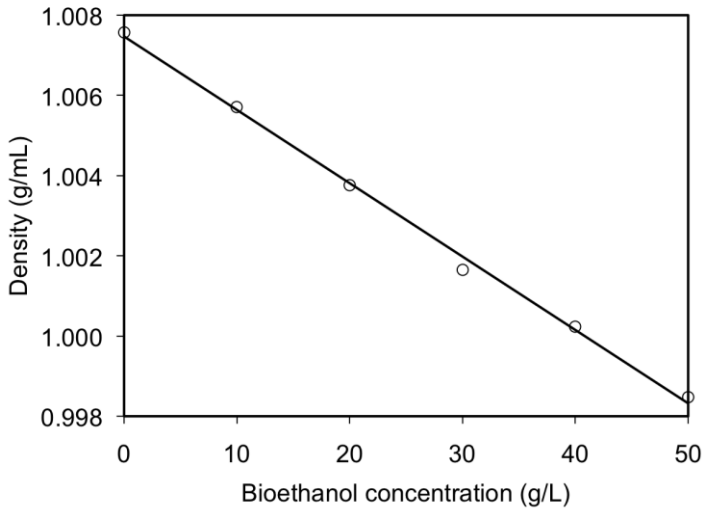

Fig. 1. Calibration curve using coffee mucilage as medium

Table 1 shows the values for initial and consumed sugar amounts, bioethanol concentrations determined by the pycnometer method and HPLC technique, and their respective standard deviations.

Table 1. Bioethanol Production from Coffee Mucilage

	x_1 (g/L)	x_2 (g/L)	y_1 (pycnometer) (g/L)		y_2 (HPLC) (g/L)	
Test 1	26.486	23.969	12.010	± 1.268	11.147	± 1.712
Test 2	49.043	45.889	18.243	± 0.510	17.570	± 0.474
Test 3	68.535	65.051	25.822	± 0.609	24.777	± 1.775
x_1: reducing sugar concentration, x_2: sugar consumption, y_1: bioethanol concentration by pycnometer method, y_2: bioethanol concentration by HPLC						

The sugar consumption was higher than 90%, and the yields of sugar in bioethanol were 45.345%, 37.198%, and 37.677% grams of bioethanol per grams of initial sugar. The fractional or relative yields were 88.740%, 72.794%, and 73.731% with initial sugar concentrations of 26.486 g/L, 49.043 g/L, and 68.535 g/L respectively. So a lower initial sugar concentration resulted in a higher yield (Fig. 2).

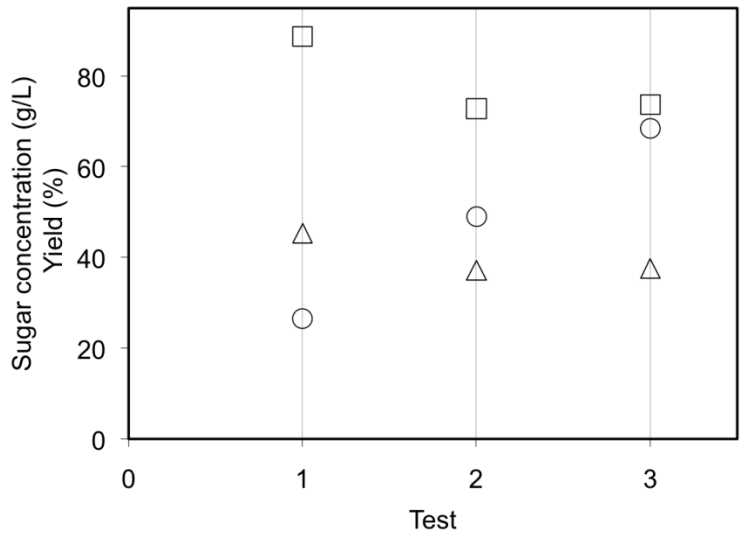

Fig. 2. Initial sugar concentrations (○), yields (△), and fractional yields (□), Pycnometer method

The results from the study of a new analytical method can be contrasted by comparison with those obtained using a second method as a reference. There are 4 degrees of freedom for all cases, so that the critical value is $t_4 = 3.36$ (P=0.05). The observed values were t (= 1.130, 1.532, and 1.418) which were less than the critical value, thereby accepting the null hypothesis. The two methods provided the same result. It was also shown that P (t>1.130) = 0.322, P (t>1.532) = 0.200, and P (t>1.418) = 0.229. Since these probabilities are greater than 0.05, the result was not significant at the 5% level; in other words, (x_1 - x_2) was not different from zero.

The F contrast for comparison of standard deviations was used to determine whether the methods differed in their precision, resulting in $F_{2,2}$= 19. The calculated value was less than this (Table 2), so there was no significant difference between the two variances at a level of 5%.

Table 2. F Contrast: Two Samples Supposing Same Variances

Bioethanol Concentration	Test 1		Test 2		Test 3	
	PM	HPLC	PM	HPLC	PM	HPLC
Mean	12.010	11.147	18.243	17.570	25.822	24.777
Variance	1.608	2.931	0.51	0.474	0.609	1.775
Remarks	3	3	3	3	3	3
Joint variance	2.269		0.242		0.112	
Degree of freedom	4		4		4	
Statistic *t*	1.130		1.532		1.418	
F-value	1.823		1.158		8.495	
PM: Pycnometer method						

The equation describing the relationship shown in Fig. 3 is as follows:

$$y_1 = 7.42e-02x_1^2 - 2.675e-02x_1 + 8.16 \quad (3)$$

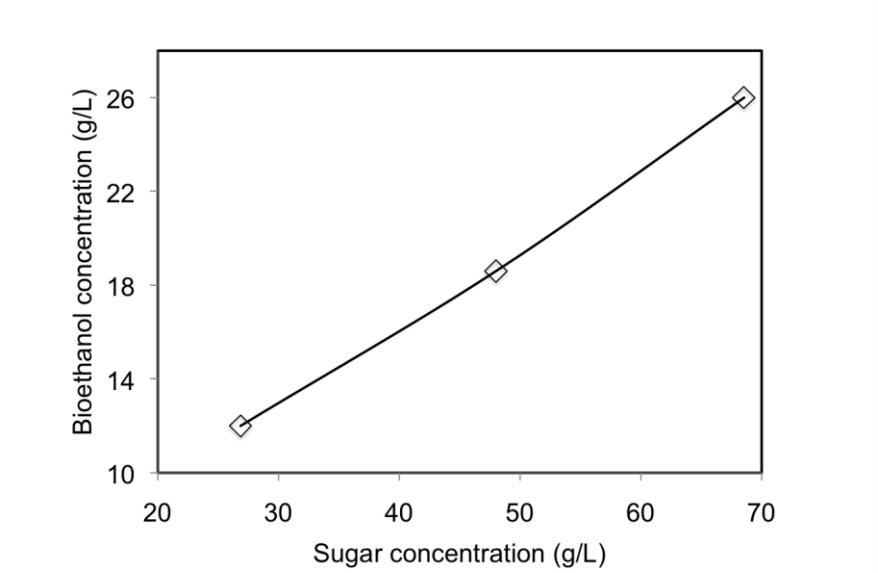

Fig. 3. Function describing equation 1 for the sugar concentration against the bioethanol concentration (Design Expert 7.0.0)

The analysis model to one factor design was significant. There was only a 0.01% chance that a model *F*-value this large could occur due to noise. Values of $P > F$ less than 0.0500 indicate that the model terms are significant. In this case, x_1^2 and x_1 were significant model terms. The adequacy of the models was expressed by the coefficient of determination (R^2), which was very close to 1.0 for determination of bioethanol concentration.

CONCLUSIONS

1. This study established and developed a methodology to determine bioethanol concentration from coffee mucilage by a pycnometer method that was verified with HPLC.
2. Initial sugar concentrations of 26.486 g/L, 49.043 g/L, and 68.535 g/L generated yields of 0.453, 0.371, and 0.376 grams of bioethanol per gram of initial sugar and fractional or relative yields of 88.740%, 72.794%, and 73.731%, respectively.
3. According to significance tests comparing the two experimental means, the pycnometer method and HPLC provided the same bioethanol concentration (pycnometer method: 12.010 g/L, 18.242 g/L, and 25.821 g/L; HPLC: 11.146 g/L, 17.570 g/L, and 24.776 g/L) with joint variances of 2.269, 0.242, and 0.112 to three different tests with initial sugar concentrations of 26.486 g/L, 49.043 g/L, and 68.535 g/L, respectively.
4. The pycnometer method proposed here is an alternative way to quantify bioethanol concentration in a fermentation medium when there is a lack of sophisticated equipment, such as is needed for HPLC.

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REFERENCES CITED

AOAC. (1990). "Official Methods, 945.06," *Official Methods of Analysis of the Association of Official Analytical Chemists*. Fifteenth edition.

Breisha, G. Z. (2010). "Production of 16% ethanol from 35% sucrose," *Biomass and Bioenergy* 34, 1243-1249. DOI: 10.1016/j.biombioe.2010.03.017

Chuck-Hernandez, C., Perez-Carrillo, E., and Serna-Saldivar, S. O. (2009). "Production of bioethanol from steam-flaked sorghum and maize," *Journal of Cereal Science* 50, 131-137. DOI: 10.1016/j.jcs.2009.04.004

Cortright, R. D., Davda, R. R., and Dumesic, J. A. (2002). "Hydrogen from catalytic reforming of biomass-derived hydrocarbons in liquid water," *Letters to Nature* 418, 964-967. DOI: 10.1038/nature11891

Ferreira, S., Gil, N., Queiroz, J., Duarte, P., and Domingues, F. C. (2010). "Bioethanol from the Portuguese forest residue *Pterospartum tridentatuman* - An evaluation of pretreatment strategy for enzymatic saccharification and sugars fermentation," *Bioresource Technology* 101(20), 7797-7803. DOI: 10.1016/j.biortech.2010.05.038

Huang, Y., Jin, Y., Fang, Y., Li, Y., and Zhao, H. (2013). "Simultaneous utilization of non-starch polysaccharides and starch and viscosity reduction for bioethanol fermentation from fresh *Canna edulis* Ker. tubers," *Bioresource Technology* 128, 560-564. DOI: 10.1016/j.biortech.2012.09.134

Karagöz, P., Rocha, I.V., Özkan, M., and Angelidaki, I. (2013). "Alkaline peroxide pretreatment of rapeseed straw for enhancing bioethanol production by Same Vessel Saccharification and Co-Fermentation," *Bioresource Technology* 104, 349-357. DOI: 10.1016/j.biortech.2011.10.075

Lachenmeier, D. W., Godelmann, R., Steiner, M., Ansay, B., Weigel, J., Krieg, G. (2010). "Rapid and mobile determination of alcoholic strength in wine, beer and spirits using a flow-through infrared sensor," *Chemistry Central Journal* 4, 1-10. DOI: 10.1186/1752-153X-4-5

Liebmann, B., Friedl, A., and Varmuza, K. (2008). "Determination of glucose and ethanol in bioethanol production by near infrared spectroscopy and chemometrics," *Analytica Chimica Acta* 642(1), 171-178. DOI: 10.1016/j.aca.2008.10.069

Miller, G. (1959). "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Analytic Chemistry* 31(1), 426-428. DOI: 10.1021/ac60147a030

Miller, J. N., and Miller, J. C. (2002). *Estadística y Quimiometría para Química Analítica*, 4th Ed., Trans. C. M. Jimenez and R.I. Hornillos, Prentice Hall, Madrid, pp. 286.

Mishima, D., Kuniki, M., Sei, K., Soda, S., Ike, M., and Fujita, M. (2008). "Ethanol production from candidate energy crops: Water hyacith (*Eichhornia crassipes*) and water lettuce (*Pistia stratiotes*)," *Bioresource Technology* 99(7), 2495-2500. DOI: 10.1016/j.biortech.2007.04.056

Mussato, S. I., Machado, E. M., Carneiro, L. M., and Teixeira, J. A. (2011). "Sugars

metabolism and ethanol production by different yeast strains," *Applied Energy* 92, 763-768. DOI: 10.1016/j.apenergy.2011.08.020

Oliver, J. D., Gaborieau, M., Castignolles, P. (2014). "Ethanol determination using pressure mobilization and free solution capillary electrophoresis by photo-oxidation assisted ultraviolet detection," *Journal of Chromatography* 1348, 150-157. DOI: 10.1016/j.chroma.2014.04.076

Pereira, F. B., Guimarães, P. M., Teixeira, J. A., and Domingues, L. (2010). "Optimization of low-cost medium for very high gravity ethanol fermentations by *Saccharomyces cerevisiae* using statistical experimental designs," *Bioresource Technology* 101(20), 7856-7863. DOI: 10.1016/j.biortech.2010.04.082

Piccolo, C. and Bezzo, F. (2009). "A techno-economic comparison between two technologies for bioethanol production from lignocellulose," *Biomass and Bioenergy* 33(3), 478-491. DOI: 10.1016/j.biombioe.2008.08.008

Pratten, N.A. (1981) "Review, The precise measurement of the density of small samples," *Journal of Materials Science* 16(7), 1737-1747. DOI: 10.1007/BF00540619

Rangel, A. O. S. S., and Tóth, I. V. (2000). "Enzymatic determination of ethanol and glycerol by flow injection parallel multi-site detection," *Analytica Chimica Acta* 416, 205-210.

Reddy, L. V. A., and Reddy, O. V. S. (2011). "Effect of fermentation conditions on yeast growth and volatile composition of wine produced from mango (*Mangifera indica* L.) fruit juice," *Food and Bioproducts Processing* 89, 487-491. DOI: 10.1016/j.fbp.2010.11.007

Rotariu, L., Bala, C., and Magearu, V. (2004). "New potentiometric microbial biosensor for ethanol determination in alcoholic beverages," *Analytica Chimica Acta* 513, 119-123. DOI: 10.1016/j.aca.2003.12.048

Shih, C. J., and Smith, E. A. (2009). "Determination of glucose and ethanol after enzymatic hydrolysis and fermentation of biomass using Raman spectroscopy," *Analytica Chimica Acta* 653, 200–6. DOI: 10.1016/j.aca.2009.09.012

Tolnai, S. (1975). "A method for viable cell count," *Methods in Cell Science* 1(1), 37-38. DOI: 10.1007/BF00914435

Vučurović, V. M., and Razmovski, R. N. (2012). "Sugar beet pulp as support for *Saccharomyces cerivisiae* immobilization in bioethanol production," *Industrial Crops and Products* 39, 128-134. DOI: 10.1016/j.indcrop.2012.02.002

Wang, M., Choong, Y., Su, N., and Lee, M. (2003). "A rapid method for determination of ethanol in alcoholic beverages using capillary gas chromatography," 11, 133-140.

Wang, W., Zhuang, X., Yuan, Z., Yu, Q., Qi, W., Wang, Q., and Tan, X. (2012). "High consistency enzymatic saccharification of sweet sorghum bagasse pretreated with liquid hot water," *Bioresource Technology* 108, 252-257. DOI: 10.1016/j.biortech.2011.12.092

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