Carbohydrate Polymers 97 (2013) 253–261

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

A new method for rapid determination of carbohydrate and total carbon concentrations using UV spectrophotometry

Ammar A. Albalasmeh, Asmeret Asefaw Berhe, Teamrat A. Ghezzehei*

Life and Environmental Sciences, University of California, Merced, United States

ARTICLE INFO

Article history:
Received 12 November 2012
Received in revised form 24 April 2013
Accepted 25 April 2013
Available online 7 May 2013

Keywords:
Phenol–Sulfuric Acid
Carbohydrate
Furfural
UV method

ABSTRACT

A new UV spectrophotometry based method for determining the concentration and carbon content of carbohydrate solution was developed. This method depends on the inherent UV absorption potential of hydrolysis byproducts of carbohydrates formed by reaction with concentrated sulfuric acid (furfural derivatives). The proposed method is a major improvement over the widely used Phenol–Sulfuric Acid method developed by DuBois, Gilles, Hamilton, Rebers, and Smith (1956). In the old method, furfural is allowed to develop color by reaction with phenol and its concentration is detected by visible light absorption. Here we present a method that eliminates the coloration step and avoids the health and environmental hazards associated with phenol use. In addition, avoidance of this step was shown to improve measurement accuracy while significantly reducing waiting time prior to light absorption reading. The carbohydrates for which concentrations and carbon content can be reliably estimated with this new rapid Sulfuric Acid–UV technique include: monosaccharides, disaccharides and polysaccharides with very high molecular weight.

1. Introduction

The determination of carbohydrate concentration in aqueous solutions is very important component of several areas of environmental research (Fukasawa, Tateno, Hagiwara, Hirose, & Osono, 2012; Goupil et al., 2012; Raynaud, Vaxelaire, Olivier, Dieude-Fauvel, & Baudez, 2012; Takei, Bartolo, Fujihara, Ueta, & Donald, 2012; Yu et al., 2012; Zhang, Banks, & Heaven, 2012) as well as industrial applications in the petroleum (Fujieda, Kitamura, Yamasaki, Furuishi, & Motobayashi, 2012; Pilavtepe, Sargin, Celiktas, & Yesil-Celiktas, 2012; Trzcinski, Hernandez, & Webb, 2012; Zhao et al., 2012; Zheng et al., 2012), pharmaceutical (Bai et al., 2012; Coura et al., 2012; Lee, Hsieh, Chen, & Chiang, 2012; Pereira et al., 2012; Zha et al., 2012), and food industries (Al-Sheraji et al., 2012; Golovchenko, Khramova, Ovodova, Shashkov, & Ovodov, 2012; Rondan-Sanabria, Valcarcel-Yamani, & Finardi-Filho, 2012; Sheu & Lai, 2012; Vriesmann, Teofilo, & de Oliveira Petkowicz, 2012). The wide diversity of carbohydrates involved in these areas has led to the development of numerous analytical techniques for measuring carbohydrate concentrations including chromatography (Jahnel, Ilieva, & Frimmel, 1998; Mason & Slover, 1971; Prodolliet et al., 1995), capillary electrophoresis (Cortacero-Ramirez, Segura-Carretero, Cruces-Blanco, de Castro, & Fernandez-Gutierrez, 2004; ElRassi & Mechref, 1996; Soga & Serwe, 2000), infrared (IR) spectroscopy (Cadet, 1999; Robert & Cadet, 1998; Wang, Liu, Li, Chang, & Jing, 2011), light scattering detection (Suortti, Gorenstein, & Roger, 1998; Zhang, Sheng, & Yu, 2008) and Nuclear Magnetic Resonance (NMR) spectroscopy (Copur, Kiemle, Stipanovic, Koskinen, & Makkonen, 2003; Duquesnoy, Castola, & Casanova, 2008). Use of many of these methods requires considerable financial investment, advanced analytical skills, and time. One of the most versatile, relatively easy and cheap approaches for determination of carbohydrate concentrations is the colorimetric method based on reaction between hydrolyzed carbohydrate solution and a coloring reagent that develops color that is detectible in the visible range of the electromagnetic spectrum. Reagents commonly used for color development include phenol (C_6H_5OH) (DuBois et al., 1956), alkaline ferricyanide ($2K_4Fe(CN)_6$) (Englis & Becker, 1943), and anthrone ($C_{14}H_{10}O$) (Dreywood, 1946).

Among the colorimetric methods for carbohydrate analysis, the Phenol–Sulfuric Acid method of DuBois et al. (1956) is so far the most reliable method and has been extensively used in a wide range of fields. At the time of writing this paper, more than 23,700 distinct peer-reviewed articles (including more than 1000 peer-reviewed articles in 2012 alone) were indexed by the Web of Science® (Thommpson Reuters) as citing the methods paper of DuBois et al. (1956). The Phenol–Sulfuric Acid method depends on dehydration of hydrolyzed saccharides to furfural derivatives during reaction with concentrated sulfuric acid (Asghari & Yoshida, 2006; Bicker, Hirth, & Vogel, 2003; DuBois et al., 1956; Hung, Selkirk, & Taylor, 1982; Itagaki, 1994; Lima et al., 2010; Rao & Pattabiraman, 1989). Further reaction of the furfural derivatives

* Corresponding author. Tel.: +1 209 228 4711.
E-mail address: taghezzehei@ucmerced.edu (T.A. Ghezzehei).

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.04.072

with phenol forms colored complexes that absorb light in the visible range, with a maximum absorbance at wave-length of 490 nm (DuBois et al., 1956; Rao & Pattabiraman, 1989).

Although it is easier to use than many of the available methods, the Phenol–Sulfuric Acid method has a few serious drawbacks. First, the coloring agent used in this method, phenol, poses multiple health hazards. Phenol and its vapors are corrosive to skin, eye, and respiratory system. Repeated and prolonged contact with skin can cause dermatitis or second and third degree burns. Similarly, prolonged or repeated inhalation of phenol vapors causes lung edema. Long-term exposure to phenol also have serious impact on the central nervous system (it is a strong neurotoxin), kidneys, and liver (Budavari, 1996; Lin, Lee, Lai, & Lin, 2006; Michalowicz & Duda, 2007). Phenol is one of 126 'Priority Pollutants' currently regulated by U.S. Environmental Protection Agency (Appendix A to 40 CFR Part 423). Secondly, the result of the standard Phenol–Sulfuric Acid method is presented in terms of glucose-equivalent concentrations. This representation may have potential limitations when dealing with complex carbohydrates that are not simple polymers of glucose. Finally, the chemical reactivity of carbohydrates with the derivatization reagent (sulfuric-acid) greatly depends on whether the carbohydrates are neutral or anionic. As a result, the molar absorption coefficients can greatly vary depending on the charge of the carbohydrates analyzed (Mecozzi, 2005).

The aim of this work was to develop an alternate for the colorimetric method of DuBois et al. (1956) that alleviates the above listed drawbacks by reducing the reaction wait-time, improving accuracy of the measurements, eliminating the hazards posed by usage of phenol, and enabling direct correlation of light absorbance to total carbon concentration in aqueous solutions.

The motivation for this study was derived from the work of Itagaki (1994), who showed that aqueous solution of furfural has UV-light absorption maxima at 277 nm. Moreover, Itagaki (1994) showed that glucose and cellulose absorb UV light at 323 nm after hydrolysis by reaction with concentrated sulfuric acid. The bathochromic shift in absorption maxima from 277 nm to 323 nm was caused by the presence of sulfuric acid in solution (Hammond & Modic, 1953; Kanetake & Otomo, 1988; Premakumari et al., 2011; Srivastava & Kumar, 2007). It has been shown that the bathochromic shift generally increases with the concentration of sulfuric acid used as solvent (Itagaki, 1994; Layne, Jaffe, & Zimmer, 1963; LP, 1974).

In this paper, we will introduce a method for determination of sugar concentrations and carbon content of aqueous solutions that depends on the UV absorbance of furfural derivates produced by reaction with concentrated sulfuric acid. We will present a comparison between the standard Phenol–Sulfuric Acid method and the proposed Sulfuric Acid–UV method using aqueous solutions of neutral (glucose, fructose, sucrose, starch, dextran and actigum) and anionic (polygalacturonic acid (PGA) and xanthan) carbohydrates that are widely used in environmental research and industrial applications.

2. Materials and methods

2.1. Reagents and apparatus

All the chemicals used in the study were of analytical reagent grade. Glucose ($C_6H_{12}O_6$) was obtained from Sigma–Aldrich. Fructose ($C_6H_{12}O_6$), sucrose ($C_{12}H_{22}O_{11}$), starch ($(C_6H_{10}O_5)_n$), phenol (C_6H_6O) and potassium hydroxide (KOH) were obtained from Fischer Scientific. Concentrated sulfuric acid (H_2SO_4) was obtained from ACROS. Polygalacturonic acid (PGA) ($(C_6H_8O_6)_n$), xanthan ($(C_{35}H_{49}O_{25})_n$) and dextran ($(C_6H_{12}O_6)_n$) were obtained from MP Biomedicals. Actigum ($(C_{24}H_{40}O_{19})_n$) was obtained from Cargill company. Absorption measurements were made on a Thermo Scientific Evolution 300 UV–vis Spectrophotometer.

A stock solution of each carbohydrate was prepared by dissolving 0.1 g of dry carbohydrate in 1 L of double milipore water (DDI). Because PGA is insoluble in water, it was made soluble by addition of potassium hydroxide (KOH). It has been previously reported that 0.46 mL of KOH is required to dissolve 100 mg of PGA (Czarnes, Hallett, Bengough, & Young, 2000). However, our preliminary experiments indicated that the pH of the prepared solution is better indicator of solubility of PGA. We found that the pH of the solution has to be raised to 12.4 for complete dissolution and this procedure was used throughout this study. Various dilutions of the stock carbohydrate solutions were made by pipetting a known volume of the stock solution and completing the volume with DDI water. The concentrations that were prepared for this study are: 0, 0.01, 0.03, 0.05, and 0.07 g/L.

2.2. Analytical methods

In the paragraphs below we describe two methods that are based on light absorption in the visible and UV range: the Phenol–Sulfuric Acid Method (DuBois et al., 1956) and the proposed Sulfuric Acid–UV method, respectively.

2.2.1. Phenol–Sulfuric Acid method

This is the most widely used colorimetric method to date for determination of carbohydrate concentration in aqueous solutions (DuBois et al., 1956). The basic principle of this method is that carbohydrates, when dehydrated by reaction with concentrated sulfuric acid, produce furfural derivatives. Further reaction between furfural derivatives and phenol develops detectible color. The standard procedure of this method is as follows. A 2 mL aliquot of a carbohydrate solution is mixed with 1 mL of 5% aqueous solution of phenol in a test tube. Subsequently, 5 mL of concentrated sulfuric acid is added rapidly to the mixture. After allowing the test tubes to stand for 10 min, they are vortexed for 30 s and placed for 20 min in a water bath at room temperature for color development. Then, light absorption at 490 nm is recorded on a spectrophotometer. Reference solutions are prepared in identical manner as above, except that the 2 mL aliquot of carbohydrate is replaced by DDI water. The phenol used in this procedure was redistilled and 5% phenol in water (w/w) was prepared immediately before the measurements.

2.2.2. Sulfuric Acid–UV method

The procedure of the proposed Sulfuric Acid–UV method is as follows. A 1 mL aliquot of carbohydrate solution is rapidly mixed with 3 mL of concentrated sulfuric acid in a test tube and vortexed for 30 s. The temperature of the mixture rises rapidly within 10–15 s after addition of sulfuric acid. Then, the solution was cooled in ice for 2 min to bring it to room temperature. Finally, UV light absorption at 315 nm is read using UV spectrophotometer. Reference solutions are prepared following the same procedure as above, except that the carbohydrate aliquot is replaced with DDI water.

2.2.3. Total carbon analysis

One of the goals of this work was to determine if there is a linear relationship between the spectrophotometric absorption of the sugar solutions and the total carbon concentration of the aqueous solutions. For this purpose, the carbon concentration of all the sugars was measured using a Shimadzu TOC-Vcsh analyzer. However, total carbon analysis of xanthan and actigum solutions using the TOC analyzer was not reliable. Because these carbohydrates form viscous suspensions, the small volume of sample extracted by the

TOC analyzer needle is not necessarily representative. Therefore, we approximated the carbon content of these solutions as:

$$[C] = \frac{n \ M_C}{M_S} p \ \ S \quad (1)$$

where [C] is theoretically calculated total carbon (in mass/volume), n is the number of C atoms in the basic unit of the carbohydrate molecule, M_C is molar mass of carbon atom, M_S is molar mass of a single unit of the carbohydrate molecule, p is the purity of the carbohydrate reagent used expressed as fraction, and S is the as-prepared concentration of the carbohydrate solution (in mass/volume).

2.3. Interaction time

We tested the effect of interaction time on the accuracy of both methods. This was done by varying the wait time after concentrated sulfuric acid is added to the carbohydrate solutions. Effect of time was tested on 0.01 and 0.07 g/L glucose solutions and wait times of 5, 15, 30, 45, 75, 105, 135 and 225 min.

2.4. Method validation

The validation of the new method (Sulfuric Acid–UV method) was performed according to the International Conference on Harmonisation (ICH) guidelines (ICH Harmonized Tripartite Guidelines., 2005). Validation process was performed in terms of the following metrics: limit of detection (LOD), limit of quantification (LOQ), linearity, precision, and accuracy. In addition, the new method was tested for possible interference from solution components that absorb in the UV range of interest, primarily proteins and flavonoids.

The limit of detection (LOD) is the lowest analyte concentration that can be detected but not necessarily quantified as an exact value, where as the limit of quantification (LOQ) is the lowest analyte concentration that can be measured with suitable precision and accuracy (Currie, 1999). LOD and LOQ are calculated for carbohydrate concentration versus absorbance and total carbon versus absorbance relationships as

$$\text{LOD} = 3.52\sigma_b \quad (2)$$

$$\text{LOQ} = 16.67\sigma_b \quad (3)$$

where σ_b is the respective standard deviation of the blank.

The linearity of an assay refers to the ability of the assay to obtain response values that are related to the analyte concentration by a defined mathematical function. In quantitative terms, linearity is expressed as the regression coefficient for fitting the data points to a straight line. The linearity was evaluated by the least square regression method with triplicate determinations at each concentration level.

The accuracy of an analytical procedure describes how well the measured concentrations agree with accepted reference values. Accuracy was assessed in terms of the percentage relative error and mean percentage recovery. For this purpose, a separate set of triplicate samples were prepared and their concentrations were determined using the fitted calibration equations. Percent relative error (δ) was determined as

$$\delta = 100\frac{[C]^* - [C]}{[C]} \quad (4)$$

where $[C]^*$ denotes carbohydrate concentration (carbon content) determined by the new method and $[C]$ is the prepared carbohydrate concentration (carbon content). Similarly, percent recovery (r) was calculated as

$$r = 100\frac{[C]^*}{[C]} \quad (5)$$

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.The precision of an analytical procedure is usually expressed in terms of the standard deviation (SD) within the series of measurements.

To test for the possible interference from the presence of proteins and/or flavonoids in sample solutions, we tested the UV absorbance of bovine serum albumin (BSA) and cinnamic acid solutions that were subjected to the full procedure of the proposed Sulfuric Acid–UV method. The objective of this test was to check whether the reaction with concentrated sulfuric acid would reduce or eliminate the UV absorbance of these compounds that are known to absorb UV light in the target range.

2.5. Statistical analysis

All measurements in this study were conducted on three replicate samples. All reported data points and spectra denote the means of the replicates. Error bars are not shown in the plots because the clutter makes it difficult to distinguish between the symbols that represent different carbohydrates. The mean and standard error of all the data reported in figures are provided in the electronic supplementary data. Data analysis was conducted using the statistical analysis software program R. Significant differences among means were analyzed by Tukey's HSD (honestly significant difference) test at probability level $\alpha < 0.05$.

3. Results and discussion

Eight carbohydrates were tested in the study, including monosaccharides (glucose and fructose), disaccharide (sucrose), and polysaccharides (starch, actigum, dextran, PGA and xanthan). PGA and xanthan are inherently anionic carbohydrates while all the others are neutral.

3.1. Absorption spectra

Light absorption in the entire visible range of the electromagnetic spectrum for all the carbohydrate solutions prepared according to Phenol–Sulfuric Acid method (DuBois et al., 1956) is presented in Fig. A1 (Supplementary data). The figures depict the fraction of the light absorbed (y-axis) as a function of wave length (x-axis). All of the carbohydrates, with the exception of PGA, have absorption maximum at 490 nm, in agreement with the original method (DuBois et al., 1956; Rao & Pattabiraman, 1989). All concentrations of PGA showed peak absorption at 478 nm. However, in the remainder of this paper, the standard 490 nm absorption is used consistently for PGA as well as the rest of the carbohydrates.

Similarly, UV light absorption in the full UV light spectrum for all the carbohydrate solutions prepared according to Sulfuric Acid–UV method is presented in Fig. A2 (Supplementary data). All of the carbohydrates, with the exception of PGA, showed maximum absorption at 315 nm. Note that this absorption peak is slightly smaller than that reported by Itagaki (1994) (322 nm). This slight bathochromic shift is related to differences in sulfuric acid concentration in the analyzed aliquots. All concentrations of PGA had maximum absorption at 297 nm. However, in the remainder of this paper, absorption at 315 nm is used for PGA in order to be consistent with the rest of the carbohydrates.

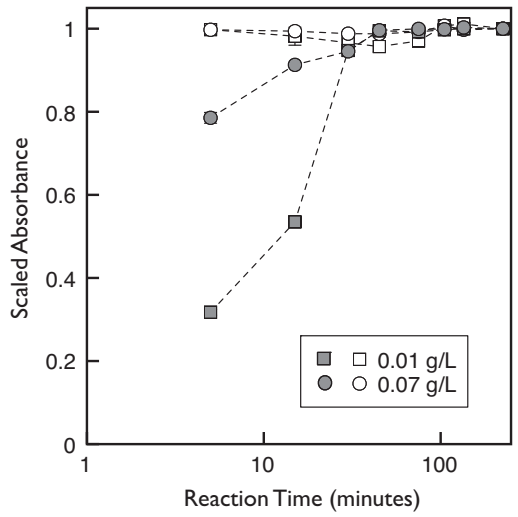

Fig. 1. Comparison the effects of reaction time on the absorbance for Phenol–Sulfuric Acid method (filled symbols) and the Sulfuric Acid–UV Method (open symbols) for two concentrations of glucose. Note that the scaled absorbance denotes the absorbance at each reaction time normalized by the absorbance at 225 min, for the respective method and concentration.

3.2. Effects of reaction time

The Phenol–Sulfuric Acid method requires several minutes for visible color development. In contrast, the proposed Sulfuric Acid–UV method is based on UV light absorption of the dehydrated carbohydrate, which requires only several seconds to be completed. Thus, one of the attractive features of the proposed method is time saving.

In Fig. 1, the reaction time after addition of sulfuric acid was added to the carbohydrate solution is compared with the scaled absorbance for both methods. The scaled absorbance was calculated by dividing the absorbance at each time by the absorbance at 225 min, which we consider as the stable reading. From these results it is evident that the Phenol–Sulfuric Acid method requires >30 minutes for full coloration of the furfural derivatives by reaction with phenol. Similar wait time recommendation was previously reported by DuBois et al. (1956) and Rao and Pattabiraman (1989). In contrast, the UV absorption of the furfural in the proposed method reaches stable level rapidly as soon as the reaction between the carbohydrates and the concentrated sulfuric acid is completed (most likely within a few seconds). Our observations are consistent with those of Itagaki (1994) who reported that UV absorbance of furfural does not change over time, remaining stable for up to five days.

3.3. Measurement of carbohydrate concentrations

Fig. 2 shows the relationship between the absorbance (x-axes) and the concentration of different carbohydrates in g/L (y-axis) for both the Phenol–Sulfuric Acid method (Fig. 2a) and the Sulfuric Acid–UV method (Fig. 2b). In Table 1 we show the coefficients of linear regression lines individually fitted to all the carbohydrate data plotted in Fig. 2 for both methods.

Before further discussing the merits of the proposed method, it is important to ascertain that the measured absorbance is indeed dependent only on the solution concentration. This concept is mathematically summarized in the Beer–Lambert Law that relates the measured absorbance to solution properties and the sample geometry

$$A = \epsilon[C]l \tag{6}$$

where A is the absorbance, ϵ [L^2/M] is the mass absorptivity, C [M/L^3] is the concentration and l [L] is the path length of the sample. We chose to use mass absorptivity (instead of molar absorptivity) because the molar mass of some of the carbohydrates used in this study (including actigum, dextran, PGA, starch and xanthan) is known only as a range of values. Eq. (6) was fitted to all the individual samples that were analyzed. The mean and coefficient of variation of the calculated mass absorptivity values are reported in Table 1 for each carbohydrate as well as for the neutral and anionic carbohydrates collectively. The coefficient of variation is generally low indicating that Beer–Lambert law is obeyed.

The results in Fig. 2 and in Table 1 clearly show that there is strong linear correlation between the carbohydrate concentrations and light absorbance, measured using both methods. Moreover, these results indicate that there is distinct difference in the concentration–absorbance relationships between the neutral carbohydrates and the anionic carbohydrates, as indicated by slopes of the regression fits to the neutral and anionic carbohydrates (reported as lines in Fig. 2). Similar difference in absorption coefficients between neutral and anionic carbohydrate for the Phenol–Sulfuric Acid method was also noted previously by Mecozzi (2005). These observations suggest that the rate of conversion from carbohydrate to furfural derivatives, upon dehydration by sulfuric acid, is not the same for neutral and anionic carbohydrates. Thus,

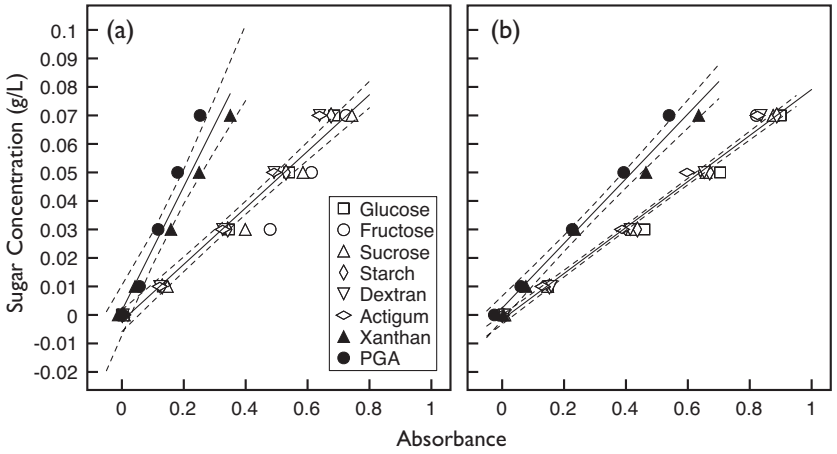

Fig. 2. Absorbance response to different carbohydrate concentrations using the (a) Phenol–Sulfuric Acid method and (b) Sulfuric Acid–UV method. The solid lines represent linear regression fit to the data and the broken lines represent 95% confidence interval of the regression line. Note that the filled symbols represent the anionic sugars while the opened symbols represent the neutral sugars. For the statistical data see Table 1.

Table 1
Coefficients of the standard curves obtained for the carbohydrates (Fig. 2) (corresponding to the absorbance versus the carbohydrate concentrations).

Sugar	Method	Slope	Intercept	Regression coefficient (R^2)	Mass absorptivity ($cm^2\ g^{-1}$)**
Glucose	Phenol–Sulfuric Acid	0.1009^{AB}	−0.0024	0.992	11.34 (0.13)
	Sulfuric Acid–UV	0.0762^{a}	−0.0017	0.992	14.25 (0.07)
Fructose	Phenol–Sulfuric Acid	0.0903^{A}	−0.0034	0.947	13.07 (0.18)
	Sulfuric Acid–UV	0.0833^{a}	−0.0017	0.992	13.11 (0.08)
Sucrose	Phenol–Sulfuric Acid	0.0939^{AB}	−0.0033	0.988	12.57 (0.14)
	Sulfuric Acid–UV	0.0802^{a}	−0.0018	0.998	13.58 (0.07)
Starch	Phenol–Sulfuric Acid	0.1028^{AB}	−0.0025	0.992	11.17 (0.13)
	Sulfuric Acid–UV	0.0786^{a}	−0.0018	0.996	13.98 (0.08)
Dextran	Phenol–Sulfuric Acid	0.1098^{AB}	−0.0028	0.993	10.51 (0.13)
	Sulfuric Acid–UV	0.0836^{a}	−0.0026	0.994	13.66 (0.12)
Actigum	Phenol–Sulfuric Acid	0.1158^{B}	−0.0025	0.981	9.73 (0.11)
	Sulfuric Acid–UV	0.0851^{a}	−0.001	0.998	12.43 (0.05)
Xanthan*	Phenol–Sulfuric Acid	0.1933^{C}	0.0015	0.998	4.88 (0.09)
	Sulfuric Acid–UV	0.108^{b}	0.0014	0.995	8.46 (0.10)
PGA*	Phenol–Sulfuric Acid	0.2888^{C}	−0.0032	0.994	4.20 (0.24)
	Sulfuric Acid–UV	0.1232^{b}	0.0026	0.999	7.28 (0.12)
Neutral	Phenol–Sulfuric Acid	0.2172	−0.0021	0.959	11.40 (0.16)
	Sulfuric Acid–UV	0.0807	−0.0016	0.990	13.50 (0.09)
Anionic	Phenol–Sulfuric Acid	0.2172	0.0016	0.935	4.54 (0.18)
	Sulfuric Acid–UV	0.1135	0.0024	0.983	7.78 (0.13)

Values within each method followed by the same letter are not significantly different at the $\alpha = 0.05$ probability level according to Tukey's honestly significant difference (HSD) test.

* Anionic carbohydrate.

** Mean (coefficient of variation (CV)).

the original Phenol–Sulfuric Acid method (DuBois et al., 1956), which uses glucose solutions as calibration standards, may not be directly applicable for anionic carbohydrates without appropriate adjustment for the calibration curves (Mecozzi, 2005).

A closer look at Fig. 2a and the slopes of the regression lines of the Phenol–Sulfuric Acid method (Table 1) reveals that there were statistically significant differences, albeit small, amongst some of the standard curves of the neutral carbohydrates. In contrast, there was no statistically significant difference amongst the regression slopes of the neutral carbohydrates measured using the Sulfuric Acid–UV method (Fig. 2b). These differences are also reflected in the coefficient of determination (R^2) values of the regression lines fitted to all the neutral and anionic carbohydrates separately (reported as lines in Fig. 2). For both methods, there were no differences in regression slopes between the pair of anionic sugars. In addition, note that the separation between the neutral and anionic regression lines is smaller in the proposed Sulfuric Acid–UV method.

The above observations are consistent with the fact that all the neutral carbohydrates are broken down to similar base sugar molecules when hydrolyzed. The statistically significant variability amongst the neutral carbohydrates, when measured using the Phenol–Sulfuric Acid method, is probably a result of slight inconsistencies in the coloration of the furfural derivatives by Phenol. Thus, the significantly better consistency of the proposed Sulfuric Acid–UV method can be explained by (a) direct dependence on the UV absorption potential of the furfural derivatives and (b) avoidance the need for a separate color development process.

Finally, the 95% confidence intervals of the grouped regression fits indicate that the maximum errors of the Phenol–Sulfuric Acid method (computed at the mid-point of the measured absorbance range for each carbohydrate group and method) are ±6.5% and ±14.4% for the neutral and anionic carbohydrates, respectively. The corresponding error levels of the proposed Sulfuric Acid–UV method are ±2.8% and ±7.5%, respectively. Thus, the proposed method cuts the measurement error by as much as half.

3.4. Measurement of total carbon content

In many applications, the total carbon content, rather than the bulk carbohydrate concentration, is the more desirable measure. To this end, we tested whether the absorbances measured according to the Phenol–Sulfuric Acid method and the proposed Sulfuric Acid–UV method are strongly correlated with total carbon content of the solutions. The carbohydrate concentrations reported in Fig. 2 were converted to total carbon content using two approaches: (a) approximated from the carbohydrate composition and solution concentration according to Eq. (1) and (b) direct measurement of the carbon content of all the solutions using TOC analyzer. The calculated and measured total carbon content values of all the carbohydrate solutions, except those of actigum and xanthan, were in strong agreement as indicated by the tight distribution of the data points around the 1:1 line in Fig. 3a. However, the measured carbon concentrations of actigum and xanthan suspensions were significantly lower than the calculated values. Similar observations were also noted byother researchers (Fontes, Queiroz, Longo, & Antunes, 2005) who analyzed total carbon content of suspensions. Likely causes for this underestimation of measured carbon content include (a) the suspended carbohydrates settle prior to extraction by the auto-sampler of the TOC analyzer and/or (b) the sampling needle is too fine to extract representative sample from the suspension. Therefore, the calculated total carbon content values of actigum and xanthan are used in the remainder of the discussions.

In Fig. 3b, the carbon content (mg/L) of the carbohydrate solutions is compared with the corresponding total carbohydrate concentrations. The coefficients of linear regression equations fitted individually to all the carbohydrates in Fig. 3b are provided in Table 2. The very high coefficients of determinations ($R^2 \geq 0.998$) of the regression fits are indicative of the precision with which the samples used in this study were prepared and the analyses were performed. It is also important to note that there were small but statistically significant differences among the slopes of the regression equations given in Table 2. Thus, there is no universal rule for conversion from total carbohydrate concentration to carbon content (and vice versa).

By combining the data reported in Figs. 2 and 3b, we derived relationships between the total carbon content of the carbohydrate solutions and absorbance values measured using the Phenol–Sulfuric Acid and Sulfuric Acid–UV methods (Fig. 4). The coefficients of linear regression equations fitted individually to all the carbohydrates are reported in Table 3. Comparison of the

Table 2
Coefficients of the measured total carbon content measurements obtained for different carbohydrate concentrations (Fig. 3B) (corresponding to the total carbon content in mg/L versus the carbohydrate concentration).

Sugar	Slope	Intercept	Regression coefficient (R^2)
Glucose	400.05^{cd}	0.7065	1
Fructose	381.39^{cd}	1.4205	0.999
Sucrose	395.95^{c}	1.2516	0.998
Starch	414.89^{c}	0.6514	1
Dextran	362.83^{ab}	0.9359	1
Actigum*	410.1^{bd}	0	1
Xanthan*	421.83^{cd}	0	1
PGA	354.95^{a}	0.8412	1

Values followed by the same letter are not significantly different at the $\alpha = 0.05$ probability level according to Tukey's honestly significant difference (HSD) test.
* Calculated total carbon.

coefficients of determination of individual carbohydrates reported in Table 1 with those in Table 3 reveals that there was no gain or loss of measurement accuracy for either method.

In addition, linear regression lines were fitted to the neutral and anionic groups of carbohydrates. The maximum errors of the Phenol–Sulfuric Acid method at 95% confidence level (computed at the mid-point of the measured absorbance range for each carbohydrate group and method) are ±6.1% and ±8.4% for the neutral and anionic carbohydrates, respectively. The corresponding error levels of the proposed Sulfuric Acid–UV method are ±2.9% and ±4.2%, respectively. It is important to highlight the fact that the error level of the proposed Sulfuric Acid–UV method is half of the Phenol–Sulfuric Acid method. In addition, note that the error in both methods is significantly diminished when used for measuring carbon content as opposed to carbohydrate concentration (Fig. 2) of the anionic solutions.

3.5. Method validation

Analytical characteristics of the Sulfuric Acid–UV method were evaluated in terms of LOD, LOQ, linearity, accuracy and precision. These validation tests were performed using the combined calibration equations that were fitted to the absorbance vs. sugar concentration and absorbance vs. carbon content data of the neutral as well as anionic sugars collectively. The LOD and LOQ for the sugar concentration calibration equations calculated using Eqs. (2) and (3) are 0.002 and 0.01 g/L, respectively. From these results, it is readily apparent that the LOD values are an order of magnitude lower than the lowest concentration used for the calibrations. The LOQ on the other hand is equal to the lowest concentration used in the calibrations. Therefore, the new method and the provided calibrations

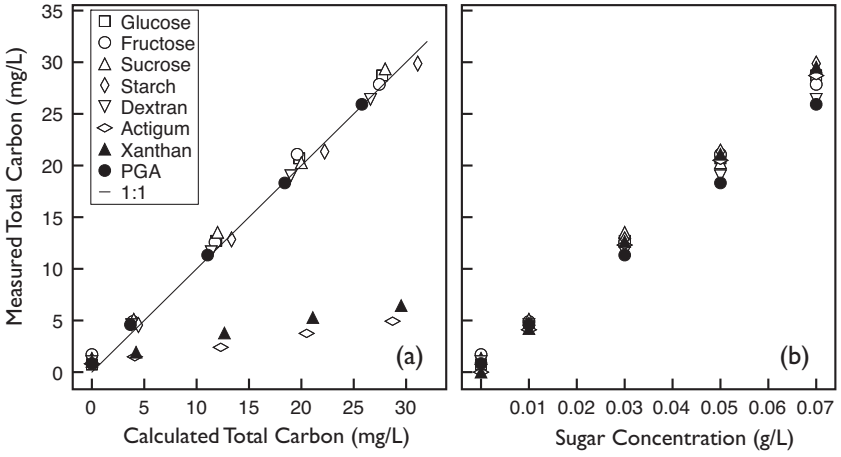

Fig. 3. Relationship between the calculated and measured total carbon for the different carbohydrate used (a) and the correlation between the total measured carbon content and carbohydrate concentrations (b). Note that calculated carbon content is used for actigum and xanthan in part (b) because the measured carbon content was unreliable (see text for explanation).

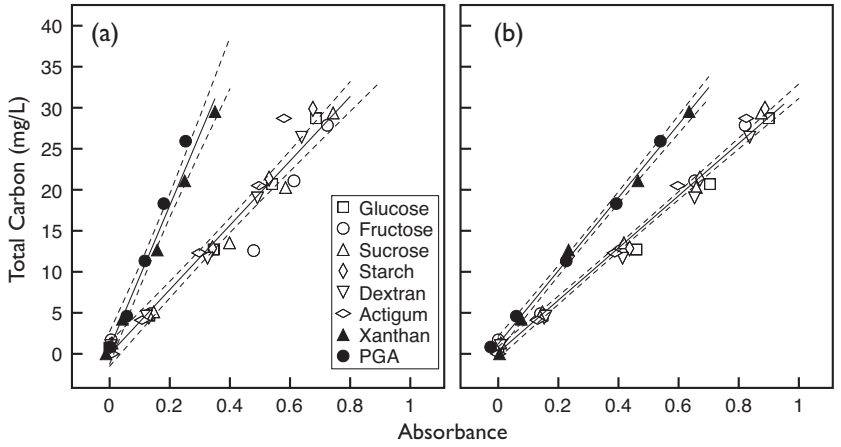

Fig. 4. Absorbance response to total carbon content for different carbohydrate concentrations using Phenol–Sulfuric Acid method (a) and Sulfuric Acid–UV method (b). The solid lines represent linear regression fit to the data and the broken lines represent 95% confidence interval of the regression line. Note that the filled symbols represent the anionic sugars while the opened symbols represent the neutral sugars. For the statistical data see Table 3.

Table 3
Coefficients of the standard curves obtained for the carbohydrates (Fig. 4) (corresponding to the absorbance versus total carbon).

Sugar	Method	Slope	Intercept	Regression coefficient (R^2)
Glucose	Phenol–Sulfuric Acid	40.377^{AB}	−0.2551	0.991
	Sulfuric Acid–UV	30.464^{a}	0.0330	0.991
Fructose	Phenol–Sulfuric Acid	34.423^{A}	0.1426	0.945
	Sulfuric Acid–UV	31.810^{a}	0.7525	0.993
Sucrose	Phenol–Sulfuric Acid	37.155^{AB}	−0.0582	0.985
	Sulfuric Acid–UV	31.749^{a}	0.5364	0.995
Starch	Phenol–Sulfuric Acid	42.587^{AB}	−0.3816	0.988
	Sulfuric Acid–UV	32.565^{a}	0.0622	0.992
Dextran	Phenol–Sulfuric Acid	39.776^{AB}	−0.0550	0.991
	Sulfuric Acid–UV	30.286^{a}	0.0024	0.993
Actigum	Phenol–Sulfuric Acid	47.499^{B}	−1.0154	0.981
	Sulfuric Acid–UV	34.913^{a}	0.4228	0.998
Xanthan*	Phenol–Sulfuric Acid	81.536^{C}	0.6349	0.998
	Sulfuric Acid–UV	45.574^{b}	0.6042	0.995
PGA*	Phenol–Sulfuric Acid	102.590^{C}	−0.2869	0.995
	Sulfuric Acid–UV	43.714^{b}	1.7694	0.998
Neutral	Phenol–Sulfuric Acid	39.231	−0.0225	0.960
	Sulfuric Acid–UV	31.848	0.1701	0.988
Anionic	Phenol–Sulfuric Acid	86.733	0.7281	0.976
	Sulfuric Acid–UV	44.612	1.2159	0.994

Values within each method followed by the same letter are not significantly different at the $\alpha = 0.05$ probability level according to Tukey's honestly significant difference (HSD) test.

* Calculated total carbon.

Table 4
Accuracy and precision of the Sulfuric Acid–UV method.

Sugar	Prepared		Measured				% Recovery		% Relative error	
	Sugar conc. (g/L)	Carbon content (mg/L)	Sugar conc. (g/L)	SD*	Carbon content (mg/L)	SD*	Sugar conc.	Carbon content	Sugar conc.	Carbon content
Glucose	0.010	4.667	0.010	0.0024	4.788	0.0035	100.00	102.59	0.00	2.59
	0.030	12.717	0.031	0.0023	12.941	0.0095	103.33	101.76	3.33	1.76
	0.070	28.723	0.070	0.0010	28.260	0.0042	100.00	98.39	0.00	1.61
Starch	0.010	4.560	0.010	0.0055	4.661	0.0021	100.00	102.19	0.00	2.19
	0.030	12.850	0.031	0.0091	12.941	0.0089	103.33	100.71	3.33	0.71
	0.070	29.867	0.071	0.0064	28.993	0.0015	101.43	97.07	1.43	2.93
PGA	0.010	4.595	0.010	0.0035	4.428	0.0018	100.00	96.37	0.00	3.63
	0.030	11.330	0.029	0.0055	11.700	0.0102	96.67	103.27	3.33	3.27
	0.070	25.923	0.068	0.0064	26.823	0.0110	97.14	103.47	2.86	3.47

* Standard deviation.

are considered applicable for quantitative measurement within the the range of the concentrations used in this study; i.e. sugar concentrations of 0.01–0.7 g/L. The linearity of the sugar concentration and carbon content calibration equations are provided in Tables 1 and 3, respectively. The observed R^2 values ($R^2 \geq 0.983$ and $R^2 \geq 0.988$, respectively) are considered high for the intended purpose of the method. The accuracy and precision of the new method are reported in Table 4. Accuracy of the method was tested in terms of percent relative error (δ) and percent recovery (r), which were calculated using Eqs. (4) and (5), respectively. In general, the new method can be considered to be accurate to within 3.6%. The standard deviation (precision) of the replicate validation samples is reported in Table 4, which is generally low indicating high precision measurement. Note that the smaller SD, the better precision.

The test for interference showed that UV absorbance of BSA and cinnamic acid is reduced but not completely eliminated after hydrolysis by reaction with concentrated sulfuric acid. Therefore, it is concluded that that the proposed method is not appropriate for samples that absorb in the UV range without any pre-treatment. As a precaution it is recommended that prior to using the proposed Sulfuric Acid–UV method, samples should be pre-screened to test if they have UV absorbance.

The reduction of UV absorbance upon hydrolysis suggests that it is possible that small quantities of protein/flavonoid impurities might be admissible, if their UV absorbance is reduced below the detection limit. The admissible level of UV absorbance of untreated samples must be established by systematic analysis of wide range of proteins and flavonoids mixed with carbohydrates at varying concentrations and proportions.

4. Conclusion

The proposed Sulfuric Acid–UV method represents a major improvement to the widely used carbohydrate characterization by Phenol–Sulfuric Acid method. The advantages of the proposed method is linked to the elimination of phenol for visible coloration of the furfural derivatives and instead capitalization on the UV absorption potential of furfural. This modification not only avoids the health and environmental hazards of phenol use but also cuts measurement error by as much as half while significantly reducing measurement time. Therefore, we can conclude that the proposed Sulfuric Acid–UV method is suitable for safer and high-throughput analysis of diverse carbohydrates in research and industrial applications. However, the Sulfuric Acid–UV method is not to be used for samples that have UV absorbance before treatment as this may indicate possible interference by protein and/or flavonoid impurities.

Acknowledgements

The authors grateful acknowledge helpful review of the manuscript by Matthew Meyer. This work was supported by

USDA-NIFA-AFRI, award number: 2010-65107-20484. AAB acknowledges support from the UC Merced new faculty startup fund.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.04.072.

References

Al-Sheraji, S. H., Ismail, A., Manap, M. Y., Mustafa, S., Yusof, R. M., & Hassan, F. A. (2012). Purification, characterization and antioxidant activity of polysaccharides extracted from the fibrous pulp of Mangifera pajang fruits. *LWT-Food Science and Technolgy*, *48*(2), 291–296.

Asghari, F., & Yoshida, H. (2006). Acid-catalyzed production of 5-hydroxymethyl furfural from D-fructose in subcritical water. *Industrial and Engineering Chemistry Research*, *45*(7), 2163–2173.

Bai, Y., Zhang, P., Chen, G., Cao, J., Huang, T., & Chen, K. (2012). Macrophage immunomodulatory activity of extracellular polysaccharide (PEP) of Antarctic bacterium *Pseudoaltermonas* sp.S-5. *International Immunopharmacology*, *12*(4), 611–617.

Bicker, M., Hirth, J., & Vogel, H. (2003). Dehydration of fructose to 5-hydroxymethylfurfural in sub- and supercritical acetone. *Green Chemistry*, *5*(2), 280–284.

Budavari, S. E. (Ed.). (1996). *The Merck index – An encyclopedia of chemicals, drugs, and biologicals*. Whitehouse Station, NJ: Merck and Co., Inc.

Cadet, F. (1999). Measurement of sugar content by multidimensional analysis and mid-infrared spectroscopy. *Talanta*, *48*(4), 867–875.

Copur, Y., Kiemle, D., Stipanovic, A., Koskinen, J., & Makkonen, H. (2003). H-1-NMR spectroscopic determination of carbohydrates and yield in pine and maple pulps. *Paperi ja Puu-Paper and Timber*, *85*(3), 158–162.

Cortacero-Ramirez, S., Segura-Carretero, A., Cruces-Blanco, C., de Castro, M., & Fernandez-Gutierrez, A. (2004). Analysis of carbohydrates in beverages by capillary electrophoresis with precolumn derivatization and UV detection. *Food Chemistry*, *87*(3), 471–476.

Coura, C. O., de Araujo, I. W. F., Vanderlei, E. S. O., Rodrigues, J. A. G., Quindere, A. L. G., Fontes, et al. (2012). Antinociceptive and anti-inflammatory activities of sulphated polysaccharides from the red seaweed Gracilaria cornea. *Basic and Clinical Pharmacology and Toxicology*, *110*(4), 335–341.

Currie, L. A. (1999). Detection and quantification limits: Origins and historical overview. *Analytica Chimica Acta*, *391*(2), 127–134.

Czarnes, S., Hallett, P., Bengough, A., & Young, I. (2000). Root- and microbial-derived mucilages affect soil structure and water transport. *European Journal of Soil Science*, *51*(3), 435–443.

Dreywood, R. (1946). Qualitative test for carbohydrate material. *Industrial and Engineering Chemistry-Analytical Edition*, *18*(8), 499–499

DuBois, M., Gilles, K., Hamilton, J., Rebers, P., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*(3), 350–356.

Duquesnoy, E., Castola, V., & Casanova, J. (2008). Identification and quantitative determination of carbohydrates in ethanolic extracts of two conifers using C-13 NMR spectroscopy. *Carbohydrates Research*, *343*(5), 893–902.

ElRassi, Z., & Mechref, Y. (1996). Recent advances in capillary electrophoresis of carbohydrates. *Electrophoresis*, *17*(2), 275–301.

Englis, D., & Becker, H. (1943). Sugar analysis by alkaline ferricyanide method – determination of ferrocyanide by iodometric and other procedures. *Industrial and Engineering Chemistry Analytical Edition*, *15*(4), 262–264.

Fontes, S., Queiroz, V., Longo, E., & Antunes, M. (2005). Tubular microporous alumina structure for demulsifying vegetable oil/water emulsions and concentrating macromolecular suspensions. *Separation and Purification Technology*, *44*(3), 235–241.

Fujieda, T., Kitamura, Y., Yamasaki, H., Furuishi, A., & Motobayashi, K. (2012). An experimental study on whole paddy saccharification and fermentation for rice ethanol production. *Biomass and Bioenergy*, *44*, 135–141.

Fukasawa, Y., Tateno, O., Hagiwara, Y., Hirose, D., & Osono, T. (2012). Fungal succession and decomposition of beech cupule litter. *Ecological Research*, *27*(4), 735–743.

Golovchenko, V. V., Khramova, D. S., Ovodova, R. G., Shashkov, A. S., & Ovodov, Y. S. (2012). Structure of pectic polysaccharides isolated from onion *Allium cepa* L. using a simulated gastric medium and their effect on intestinal absorption. *Food Chemistry*, *134*(4), 1813–1822.

Goupil, P., Benouaret, R., Charrier, O., ter Halle, A., Richard, C., Eyheraguibel, B., et al. (2012). Grape marc extract acts as elicitor of plant defence responses. *Ecotoxicology*, *21*(5), 1541–1549.

Hammond, G., & Modic, F. (1953). Aromatic nitration. I. The ultraviolet spectra of aromatic nitro compounds in sulfuric acid. *Journal of the American Chemical Society*, *75*(6), 1385–1388.

Hung, C., Selkirk, A., & Taylor, R. (1982). A chromatographic quality control procedure based on hplc for 5-hydroxymethylfurfural in autoclaved D-glucose infusion fluids. *Journal of Clinical and Hospital Pharmacy*, *7*(1), 17–23.

ICH Harmonized Tripartite Guidelines. (2005). Validation of analytical procedures: Text and methodology q2 (r1).

Itagaki, H. (1994). Saccharification process of cellulose in 97-percent sulfuric-acid monitored by sulfuric-acid induced ultraviolet-absorption behavior. *Polymer*, *35*(1), 50–52.

Jahnel, J., Ilieva, P., & Frimmel, F. (1998). HPAE-PAD – A sensitive method for the determination of carbohydrates. *Fresenius Journal of analytical Chemistry*, *360*(7-8), 827–829.

Kanetake, T., & Otomo, M. (1988). Extractive spectrophotometric determination of palladium with Di-2-pyridylmethanone 2-(5-Nitro)pyridylhydrazone. *Analytical Sciences*, *4*(4), 411–415.

Layne, W., Jaffe, H., & Zimmer, H. (1963). Basicity of N-nitrosamines. 2. Aqueous sulfuric acid solutions. *Journal of the American Chemical Siciety*, *85*(12), 1816-&.

Lee, J.-G., Hsieh, W.-T., Chen, S.-U., & Chiang, B.-H. (2012). Hematopoietic and myeloprotective activities of an acidic Angelica sinensis polysaccharide on human CD34(+) stem cells. *Journal of Ethnopharmacology*, *139*(3), 739–745.

Lima, S., Antunes, M. M., Fernandes, A., Pillinger, M., Ribeiro, M. F., & Valente, A. A. (2010). Acid-catalysed conversion of saccharides into furanic aldehydes in the presence of three-dimensional mesoporous al-tud-1. *Molecules*, *15*(6), 3863–3877.

Lin, T.-M., Lee, S.-S., Lai, C.-S., & Lin, S.-D. (2006). Phenol burn. *Burns*, *32*(4), 517–521.

LP, Z. (1974). Spectrophotometric assay of long-chain unsaturated and hydroxy fatty acids in concentrated sulfuric acid. *Analytical Biochemistry*, *5*(1), 146–154.

Mason, B. S., & Slover, H. T. (1971). Gas-chromatographic method for the determination of sugars in foods. *Journal of Agricultural and Food Chemistry*, *19*(3), 551–554.

Mecozzi, M. (2005). Estimation of total carbohydrate amount in environmental samples by the phenol–sulphuric acid method assisted by multivariate calibration. *Chemometrics and Intelligent Laboratory Systems*, *79*(1-2), 84–90.

Michalowicz, J., & Duda, W. (2007). Phenols – Sources and toxicity. *Polish Journal of Environmental Studies*, *16*(3), 347–362.

Pereira, L. d. P., da Silva, R. O., de Souza Ferreira Bringel, P. H., Sales da Silva, K. E., Sampaio Assreuy, A. M., & Pereira, M. G. (2012). Polysaccharide fractions of Caesalpinia ferrea pods: Potential anti-inflammatory usage. *Journal of Ethnopharmacology*, *139*(2), 642–648.

Pilavtepe, M., Sargin, S., Celiktas, M. S., & Yesil-Celiktas, O. (2012). An integrated process for conversion of Zostera marina residues to bioethanol. *Journal of Supercritical Fluids*, *68*, 117–122.

Premakumari, J., Roy, G. A. G., Prabhu, A. A. M., Venkatesh, G., Subramanian, V. K., & Rajendiran, N. (2011). Effect of solvents and pH on beta-cyclodextrin inclusion complexation of 2,4-dihydroxyazobenzene and 4-hydroxyazobenzene. *Journal of Solution Chemistry*, *40*(2), 327–347.

Prodolliet, J., Bruelhart, M., Lador, F., Martinez, C., Obert, L., Blanc, M., et al. (1995). Determination of freeand total carbohydrate profile in soluble coffee. *Journal od AOAC International*, *78*(3), 749–761.

Rao, P., & Pattabiraman, T. N. (1989). Reevaluation of the phenol–sulfuric acid reaction for the estimation of hexoses and pentoses. *Analytical Biochemistry*, *181*(1), 18–22.

Raynaud, M., Vaxelaire, J., Olivier, J., Dieude-Fauvel, E., & Baudez, J.-C. (2012). Compression dewatering of municipal activated sludge: Effects of salt and pH. *Water Research*, *46*(14), 4448–4456.

Robert, C., & Cadet, F. (1998). Analysis of near-infrared spectra of some carbohydrates. *Applied Spectroscopy Reviews*, *33*(3), 253–266.

Rondan-Sanabria, G. G., Valcarcel-Yamani, B., & Finardi-Filho, F. (2012). Effects on starch and amylolytic enzymes during Lepidium meyenii Walpers root storage. *Food Chemistry*, *134*(3), 1461–1467.

Sheu, S.-C., & Lai, M.-H. (2012). Composition analysis and immuno-modulatory effect of okra (*Abelmoschus esculentus* L.) extract. *Food Chemistry*, *134*(4), 1906–1911.

Soga, T., & Serwe, M. (2000). Determination of carbohydrates in food samples by capillary electrophoresis with indirect UV detection. *Food Chemistry*, *69*(3), 339–344.

Srivastava, K. P., & Kumar, A. (2007). Solvent dependence of the electronic absorption spectra of mercury-phthalocyanine. *Asian Journal of Chemistry*, *19*(1), 361–365.

Suortti, T., Gorenstein, M., & Roger, P. (1998). Determination of the molecular mass of amylose. *Journal of Chromatography A*, *828*(1–2), 515–521.

Takei, Y., Bartolo, R. C., Fujihara, H., Ueta, Y., & Donald, J. A. (2012). Water deprivation induces appetite and alters metabolic strategy in Notomys alexis: Unique mechanisms for water production in the desert. *Proceedings of the Royal Society B-Biological Sciences*, *279*(1738), 2599–2608.

Trzcinski, A. P., Hernandez, E., & Webb, C. (2012). A novel process for enhancing oil production in algae biorefineries through bioconversion of solid by-products. *Bioresource Technology*, *116*, 295–301.

Vriesmann, L. C., Teofilo, R. F., & de Oliveira Petkowicz, C. L. (2012). Extraction and characterization of pectin from cacao pod husks (*Theobroma cacao* L.) with citric acid. *LWT-Food Science and Technology*, *49*(1), 108–116.

Wang, Z., Liu, X., Li, R., Chang, X., & Jing, R. (2011). Development of near-infrared reflectance spectroscopy models for quantitative determination of water-soluble carbohydrate content in wheat stem and glume. *Analytical Letters*, *44*(15), 2478–2490.

Yu, J., Lv, L., Lan, P., Zhang, S., Pan, B., & Zhang, W. (2012). Effect of effluent organic matter on the adsorption of perfluorinated compounds onto activated carbon. *Journal of Hazardous Materials*, *225*, 99–106.

Zha, X.-Q., Pan, L.-H., Luo, J.-P., Wang, J.-H., Wei, P., & Bansal, V. (2012). Enzymatic fingerprints of polysaccharides of Dendrobium officinale and their application

in identification of Dendrobium species. *Journal of Natural Medicines*, *66*(3), 525–534.

Zhang, M.-L., Sheng, G.-P., & Yu, H.-Q. (2008). Determination of proteins and carbohydrates in the effluents from wastewater treatment bioreactors using resonance light-scattering method. *Water Research*, *42*(13), 3464–3472.

Zhang, Y., Banks, C. J., & Heaven, S. (2012). Anaerobic digestion of two biodegradable municipal waste streams. *Journal of Environmental Management*, *104*, 166–174.

Zhao, M. X., Yan, Q., Ruan, W. Q., Miao, H. F., Ren, H. Y., & Xu, Y. (2012). A comparative study of sequential hydrogen-methane and independent methane production from kitchen wastes. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects*, *34*(11), 1046–1054.

Zheng, Y., Yu, C., Cheng, Y.-S., Lee, C., Simmons, C. W., Dooley, T. M., et al. (2012). Integrating sugar beet pulp storage, hydrolysis and fermentation for fuel ethanol production. *Applied Energy*, *93*(SI), 168–175.