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Development and validation of HPLC method for determination of sugars in palm sap, palm syrup, sugarcane jaggery and palm jaggery

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Abstract

A simple and reproducible method for the qualitative and quantitative analysis of free mono- (fructose, glucose) and disaccharides (sucrose) using a high performance liquid chromatography (HPLC) with refractive index (RI) detector and a NH_2 column was developed for matrices rich in monosaccharides (honey) and disaccharides (palm sap, palm syrup, sugarcane jaggery, palm jaggery). The developed HPLC method was validated in terms of their linearity, limit of detection and quantification, precision and accuracy. Method validation studies showed that 85:15 (v/v) acetonitrile/water (CH_3CN:H_2O) was suitable for matrix containing monosaccharides and 65:35 (v/v) (CH_3CN: H_2O) was suitable for matrix containing disaccharides. The method showed good linearity with determination coefficients exceeding 0.99. Recovery studies indicated that 65% (CH_3CN:H_2O) system was suitable for matrix rich in sucrose (e.g. jaggery, syrup, etc.).

Introduction

Natural sweeteners (honey, sugarcane jaggery, palm jaggery or syrup made from sugarcane juice or sap of palmyrah palm or coconut palm) vary with respect to their chemical constituents. Honey is the oldest natural sweetener known and is predominated in glucose / fructose in the ratio 1:1.2 and also contains disaccharides like sucrose, maltose etc in lower levels (Yilmaz *et al*., 2014). Unlike honey, other natural sweeteners like sugarcane jaggery, palm jaggery, syrup made from sugarcane juice or sap of palmyrah palm or coconut palm or maple tree are dominated by disaccharides like sucrose (Singh *et al*., 2011; Zhang *et al*., 2014).

Determination of the composition of low molecular weight sugars is important for characterizing physiological and biochemical processes in plants (Glyad, 2002). HPLC with refractive index detector (RID) is widely used for determining sugars and there are several columns viz. amino column, lead carbohydrate column, etc. and mobile phases recommended for the purpose (Folkes and Jordan, 1996; Pushparajah and Nicholas, 2006). In case of amino columns, a chemically modified silica gel containing bonded aminopropyl group is used as a sorbent and aqueous CH3CN is used as a solvent. The ratio between water and CH_3CN in the mobile phase depends on the nature of compounds under investigation. Researchers have reported that a mobile phase consisting of 75% CH3CN is most suitable for oligosaccharide separation, while the systems with a higher CH_3CN content must be used for monosaccharide, using lower CH_3CN content the monosaccharides coincided in their retention time (Glyad, 2002).

Method validation is a prerequisite when new matrices are studied for the analyte of interest (Rogers, 2013). Even though there are many reports on using aqueous CH3CN and amino columns for sugar separation, there are no reports which focus on method validation of sugars in matrices rich in sucrose like that of palm sap, jaggery or syrup prepared from sugarcane juice or saps from *Cocus nucifera* L or *Borassus flabellifer* L. During the course of our study on syrups and jaggery, it was found that jaggery made from sugarcane juice showed a lower value for sucrose content (65%) when 85% CH_3CN was used as the mobile phase. This necessitated changing the ratio of CH_3CN to H_2O to arrive at the best suitable concentration of mobile phase that can be used for matrices rich in sucrose. In the present study, we are thus reporting a method validation study to determine the sugar content in honey, palm sap, palm syrup and

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sugarcane jaggery and palm jaggery using HPLC-RID which warrants the suitability of choosing the HPLC conditions for matrices extremely rich in mono or disaccharides. The method was validated in terms of their linearity, limit of detection and quantification, precision and accuracy.

Materials and Methods

Chemicals and materials

HPLC grade CH3CN was obtained from Merck (Mumbai, India). HPLC water was purified on a Milli-Q-system (Millipore India Pvt Ltd, Bangalore, India).All carbohydrate standards (glucose, fructose and sucrose) were procured from Sigma-Aldrich (USA). Palm sap and palm jaggery used for the study was supplied by Trivandrum District Palm Products Development Co-operative Federation, Kerala, India. Palm syrup was prepared by the protocol developed in the lab (data not shown). Sugarcane jaggery and honey was procured from the local market in Thiruvananthapuram, Kerala, India.

Assay sample preparation and estimation of sugars using HPLC-RID

The weighed samples (1-2.5g) were dissolved in 25 mL of HPLC grade water and the solution was centrifuged on a Remi C 30 centrifuge (Mumbai, India) at 16,000 rpm for 10 minutes and the supernatant was collected and filtered through a 0.2µm nylon filter (Micro-Por Minigen Syringe Filter, Genetix Biotech Asia, New Delhi).

Sugars were analyzed using HPLC system (LC-20AD/T HPLC Series, Shimadzu, Kyoto, Japan) on a reverse phase Supelcosil LC-NH_2 column (25 cm × 4.6 mm, 5 µm), using a refractive index detector (RID-10A, Shimadzu Corporation, Kyoto, Japan) with an isocratic mobile phase of CH_3CN:H_2O (85:15, v/v) and (65:35, v/v), maintained at a flow rate 1.5 mL/min. Sample injection volume was 20 µL, and analysis was carried out at 35°C. Standard curves for fructose, and sucrose were prepared in HPLC grade water at concentrations ranging from 1-5% (w/v) and for glucose from 1-10% (w/v). All the samples and standards were analyzed in triplicate.

The chromatographic peaks corresponding to each sugar were matched with the retention time of the standard. A calibration curve fitted by linear regression analysis using origin pro 8 software was prepared using standards to determine the relationship between the peak area and concentration.

Validation of method

The developed HPLC methods were validated in terms of their linearity, limit of detection and quantification, precision and accuracy.

Linearity

Linearity was established by measuring the instrument response of a sufficient number (at least five) of standard solutions in the expected range of the analyte. It was estimated by the equation of the regression line (y = ax + b) by plotting concentrations (x) versus the response (y) (Caldas *et al*. 2009). Linearity between the detector responses and concentration of fructose (1 to 5%), glucose, (1 to 10%) and sucrose (1 to 5%) in HPLC was evaluated. All the standards showed a correlation coefficient within the range of $r^2 = 0.99$.

Limit of detection and limit of quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the sugars were calculated using the equation: LOD = 3.3 σ/S and LOQ = 10σ/S, respectively, where, σ is the standard deviation of the response and S is the slope of the corresponding calibration curve.

Precision

Precision was determined as both repeatability and intermediate precision. Repeatability of sample injection was determined as intra-day variation and intermediate precision was determined by measurement of inter-day variation. Repeatability expresses the precision under the same operating conditions over a short interval of time. Precision was determined through the calculation of the relative standard deviation (RSD), as shown in equation, where s is the standard deviation and x is the average values.

$$\% \text{ RSD} = (s/x) \times 100$$

Accuracy

Accuracy of the methods was determined by standard addition techniques. Known amounts of standards in a range of low, medium and high concentration were added to pre analyzed samples and analyzed under the optimized conditions. Addition experiments for each concentration were performed in triplicate and the accuracy was calculated as the % of analyte recovered. Three analyses per concentration were performed and mean ± SD was determined. The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness. In this work, accuracy

was determined through the percentage of recovery of known amounts of analyte added in the sample. The recovery percentage R (%) was calculated by the following equation:

$$R\ (\%) = (A_{(a+s)} - A_a / A_s) \times 100$$

Where, A_a is the concentration of actual analyte in sample, A_s is the weight of standard added and $A_{(a+s)}$ is the concentration of actual analyte in sample with standard addition. The recovery tests were performed by adding known amounts of standards (0.15, 0.3 and 0.45g/1.5g) to sample. Before the addition of standards, the samples were centrifuged and the supernatant, after being filtered through a 0.2 µm nylon filter, was analyzed by HPLC.

Results

Preparation of calibration curve

Chromatographic measurements were standardized using the absolute calibration method. Figure 1 shows the HPLC chromatograms of standards. Retention time (RT) of fructose, glucose and sucrose was 4.16, 4.83, and 6.98 min, respectively (Figure 1) when 85% CH_3CN was used. On using 65% CH3CN the separation of glucose and fructose was not possible as they eluted as single peak but better peak symmetry was achieved for sucrose (RT-2.76) which was not observed when 75% and 85% CH_3CN was used. Hence 85% CH3CN was used for separation of monosaccharides and 65% was used for separation of disaccharides. Calibration curves were prepared using a series of standard solutions of fructose, and sucrose at concentrations ranging from 1 to 5% and for glucose from 1 to 10%. Five analyses per concentration were conducted. Concentration of standards was selected based on the published data from prior art (Glyad, 2002).

HPLC profiling of carbohydrates

The sugar compositions of honey, sugarcane jaggery, palm jaggery, palm sap and palm syrup are given in Table 1 and the peaks obtained by chromatography is shown in Figure 2. Sugarcane jaggery, palm jaggery, palm sap and palm syrup showed only the presence of sucrose, whereas honey sample contained fructose (34.0%) glucose (34.43%) and sucrose (3.3%) giving a total sugar of 71.73%.

Validation of developed method

Linearity

Under the chromatographic conditions described earlier, a good linearity ($r^2 = 0.997$ for fructose, 0.999 for glucose and 0.999 for sucrose) was obtained with regression equations of y = 1158130x - 55340, y = 1194890x - 48441, y = 1120090x + 2702 for fructose, glucose, and sucrose, respectively (Table 2) for 85% CH_3CN. Calibration curve plotted for sucrose using 65% CH_3CN gave a good linearity with $r^2 = 0.999$ with regression equation of y = 1053200x-88318.

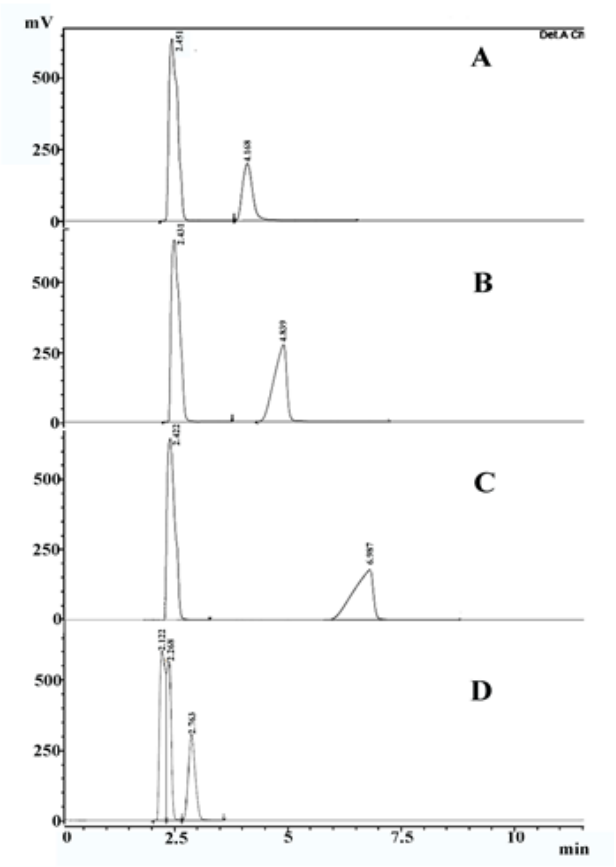

Figure 1. HPLC Chromatograms of standards using 85% CH_3CN: A. Fructose (RT- 4.1), B. Glucose (RT- 4.8), C. Sucrose (RT- 6.9), and D. Sucrose (RT- 2.7) using 65% CH_3CN

LOD and LOQ

The limit of detection is the lowest concentration of an analyte in a sample that can be detected but cannot be used for the quantification and limit of quantification is the lowest concentration of the analyte in a sample that can be quantified with acceptable precision and accuracy under the conditions of operation. LODs of fructose and glucose were found to be 0.35 (w/v) and 0.209% (w/v) respectively, and the corresponding LOQs were found to be 1.05 (w/v) and 0.635% (w/v) for fructose and glucose (Table 2). In case of sucrose, LOD and LOQ for 85% CH_3CN was 0.02 (w/v) and 0.07% (w/v) respectively and with 65% CH_3CN LOD was 0.19% (w/v) and LOQ was 0.58% (w/v).

Table 1. Total sugars in samples analyzed by HPLC-RID

Sample	Fructose (%)	Glucose (%)	Sucrose (%)	Total (%)
Honey[a]	34.0±1.1	34.43±0.5	3.3±0.2	71.73±1.8
Sugarcane jaggery[a]	ND	ND	65.0±0.8	65.0±0.8
Sugarcane jaggery[b]	ND	ND	86.02±1.0	86.02±1.0
Palm jaggery [b]	ND	ND	83.47±0.5	83.47±0.5
Palm sap [b]	ND	ND	13.8±1.2	13.8±1.2
Palm syrup [b]	ND	ND	73.94±0.8	73.94±0.8

[a] - 85 % CH3CN, [b] - 65% CH_3CN, ND-not detected. Each value in the table represents average of (± SD) of 3 replications

Precision

The intermediate precision (intra-day precision) and repeatability of system (interday precision) were checked by injecting the different concentrations of sample solution on the same day and different days, respectively. Intra and inter assay precision are shown in Table 2 in terms of %RSD. The values were below 2%, which shows that the method was precise.

Accuracy as recovery

The proposed method was applied for the determination of the fructose, glucose and sucrose from the sample. The results indicated that, the mean percent recoveries were in the range of 107.3 to 115.3% with an average value of 111.2% for fructose, 102.2 to 106.6% with an average value of 104.0% for glucose and 58.6 to 84% and average value of 69.5% for sucrose on using 85% CH_3CN system. The recovery of sucrose improved on using 65% CH3CN and the values ranged from 71% to 102% with an average value of 86.3%. Result of the recovery study is summarized in Table 3.

Discussion

Present study was aimed at developing a suitable HPLC–RID method for quantifying sugars in matrices viz. jaggery and syrup, which are rich in sucrose unlike juices and honey. Calibration curve was plotted for standards (glucose, fructose and sucrose) and linearity was determined. The linearity of an analytical method refers to the ability to obtain results either directly, or after mathematical transformation proportional to the concentration of the analyte in the sample within a given range (Shabir 2003; Chandran and Singh, 2007). The ratio of CH_3CN:H_2O was changed from 85:15 to 65:35 to check the separation of monosaccharides (glucose and fructose) and disaccharides (sucrose). This trial showed that 85% CH_3CN is the most suitable for glucose and fructose and 65% CH3CN was ideal for sucrose estimation. Further the LOD and LOQ values of the analytes were estimated. LOD and LOQ represent the lower concentration of the substance

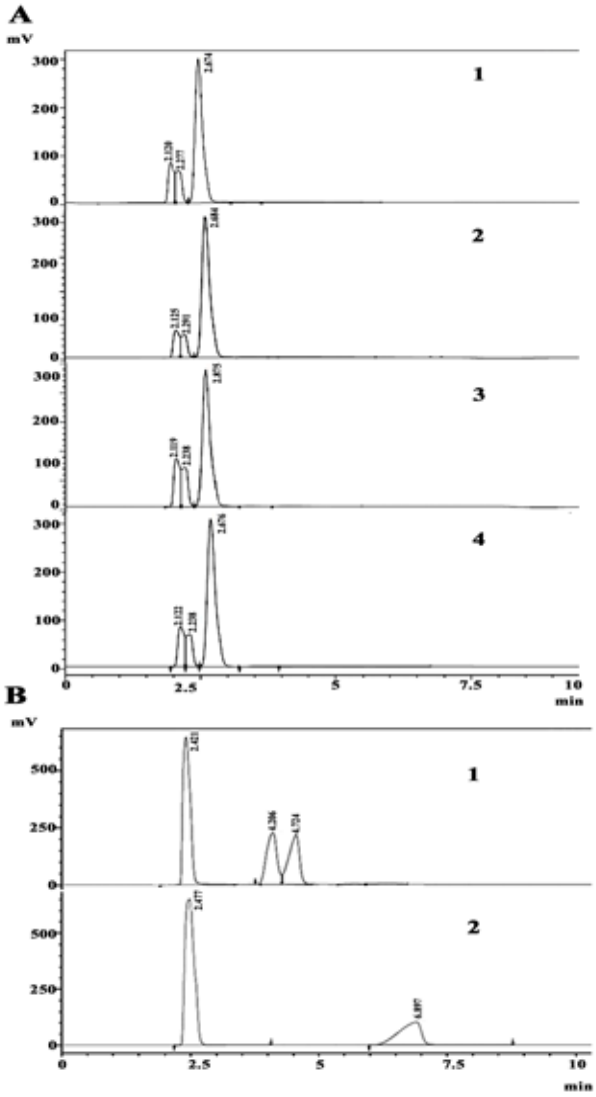

Figure 2. HPLC Chromatograms of samples using A. 65% CH_3CN: 1.Palm Syrup (Sucrose - RT- 2.6) 2. Palm jaggery (Sucrose - RT- 2.6) 3. Palm sap (Sucrose - RT- 2.8) 4. Sugarcane jaggery (Sucrose - RT- 2.6). B. 85% CH3CN: 1. Honey 2. Sugarcane jaggery (Fructose RT- 4.2; Glucose RT- 4.7; Sucrose RT - 6.8)

Table 2. Linearity, LOD and LOQ, precision of fructose, glucose and sucrose analyzed by HPLC - RID

Parameter	Fructose (85%) ACN)	Glucose (85%) ACN)	Sucrose (85%) ACN)	Sucrose (65%) ACN)
Regression equation[a]	y =1158130x-55340	y =1194890x-48441	y =1120090x+2702	y =1053200x-88318
Linear range (%)	0-5	0-10	0-5	0-5
r^2	0.997	0.999	0.999	0.999
LOD (%)	0.35	0.209	0.02	0.19
LOQ (%)	1.05	0.635	0.07	0.58
Precision				
Repeatability (%)	0.86	0.49	0.29	1.24
Intermediate precision (%)	0.29	0.20	0.05	1.00

[a] y = peak area, x = concentration. Data are mean of 3 replications

Table 3. Results of recovery studies of standards

Compound	Content (g)	Added amount (g)	Theoretical amount (g)	Recorded amount (g)	Recovery amount (g)	Average recovery (%)	RSD (%)
Fructose [a]	0.547	0.15	0.697	0.72	115.3	111.2	1.58
	0.547	0.30	0.847	0.88	111.0		1.45
	0.547	0.45	0.997	1.03	107.3		1.81
Glucose [a]	0.530	0.15	0.680	0.69	106.6	104	1.92
	0.530	0.30	0.830	0.84	103.3		1.35
	0.530	0.45	0.980	0.99	102.2		2.02
Sucrose [a]	0.042	0.15	0.192	0.13	58.6	69.5	5.09
	0.042	0.30	0.342	0.24	66.0		2.24
	0.042	0.45	0.492	0.42	84.0		1.64
Sucrose [b]	0.690	0.5	1.19	1.20	102.0	86.3	1.33
	0.690	1.5	2.19	1.98	86.0		1.72
	0.690	3.0	3.69	2.82	71.0		2.0

[a] 85% CH3CN ; [b]65% CH3CN

under evaluation that can be detected and measured, respectively, using a certain experimental procedure.

To study the reliability, suitability and accuracy of the method, recovery experiments were carried out. The recovery studies were carried out three times over the specified concentration range and amount of standards were estimated by measuring the peak area ratios by fitting these values to the straight line equation of calibration curve. From the above determination, percentage recovery and standard deviation of percentage recovery were calculated. The contents were determined from the respective chromatograms. Caldas *et al.* (2009) reported that the recovery study is an important factor in the validation to evaluate the accuracy of the analytical method. It is important to obtain high recoveries (close to 100%) with good precision and small changes in the experimental conditions should not affect the robustness of the recovery values. Recovery values vary depending on many factors including the sample matrix, sample preparation procedure, properties of the analyte of interest and its concentration. Hence, from our study it is understood that a matrix with sucrose should be estimated using 65% CH_3CN rather than 85% CH_3CN, while 85% CH_3CN is suitable for estimation of glucose and fructose.

Conclusion

The method developed in this study was used for determination of the fructos, glucose and sucrose content of honey, palm jaggery, sugarcane jaggery, palm sap and palm syrup. The method validation parameters suggest that samples dominant in sucrose shows better recovery with 65% CH_3CN. The total sugar content in jaggery was 65% when estimated with 85% CH_3CN and 86.02% on estimating with 65% CH_3CN. The result discussed agrees to the data of recovery studies which shows that the recovery of sucrose was less on using 85% CH_3CN. On observing the chromatograms, it is understood that both the solvent systems studied did not show the presence of reducing sugars (fructose and glucose) in palm syrup, palm jaggery, palm sap and sugarcane jaggery.

To the best of our knowledge there are very few reports on method validation studies of sugars. The proposed HPLC-RID method has been evaluated in terms of LOD, LOQ linearity, precision and accuracy, in a varying concentration range with $r^2 > 0.99$. Statistical findings prove that the method developed is suitable for the determination of sugars -monosaccharides and disaccharides in juices and syrups. This method helps in monitoring the possible adulteration and stability in the sugar fraction of the samples in a short time and also does not involve cumbersome sample preparation procedures.

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