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Research Article

Rapid Profiling for Sugar Estimation in Sugarcane by Using HPLC-RI and Genetic Evaluation by Using RAPD Molecular Markers

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ABSTRACT

The rapid HPLC-RI based sugars profiling method optimized and applied for the qualitative and quantitative estimation of different sugars present in sugarcane extract. The concentrations of sugars were found differentially modulated in high and low sucrose sugarcane cultivars during their active maturation phases of 300, 360 and 420 days after planting (DAP). The average decrease in fructose and glucose were estimated about 2.2 and 1.5 folds from 300- 420DAP respectively. However, the sucrose was enhanced to an average of 12.2 folds from initial to final time point. These saccharide analyses were found negative in correlation with the fructose and glucose while, positive with sucrose accumulation during maturation phase of sugarcane which was estimated by Pearson correlation. Further, the genetic evaluation was determined by using 10 RAPD primers, 40% of the primers (OPAB07, OPK07, OPK10 and OPK15) found polymorphic for the given locus. The polymorphic percentages were ranged from 17-40% obtained for OPK07 and OPK15 respectively. Total 61 alleles were amplified of which 8 alleles were found polymorphic with 13.11%. This HPLC-RI method was found significant towards the estimate of sugars in different samples with great precision and estimation in less than 12 minutes. Whereas, the sugar analysis and the RAPD markers provides major insights in complex polyploidy genome evaluation associated with the sucrose accumulation in commercial sugarcane hybrids.

KEY WORDS Brix, HPLC, sugarcane cultivars, genetic evaluation, molecular markers

Introduction

Pollution of the Sugarcane hybrid (Saccharum spp.) is an important cash crop which gives lucrative returns to growers in terms of sucrose, ethanol and allied products. It majorly cultivated in tropical and subtropical region of other countries [11].

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Present day, sugarcane hybrid cultivars are developed as an inter-specific hybrids (2n=100-300) focused to enhance sucrose and abiotic stresses resistance [11, 12]. The metabolite acting as osmo-protectent against the oxidative stresses and provides resistance to plants [32]. Despite of narrow gene pool, complex genome, poor flowers fertility and long breeding selection cycle, scientist perform physio-biochemical and genetic studies to

improve sugarcane. The photosynthetic carbon gets fixed to sucrose under controlled process of carbohydrate metabolism [28]. Eventually, in active phase of carbohydrate or saccharide synthesis, the sucrose acting as signaling molecule causes metabolic stress to tissue affects modulation of sugar and stress associated enzymes [12, 24] and genes [27]. The modulation of sugar and stress associated enzymes in high and low sucrose cultivars at different time points showed major findings about sucrose accumulation [17]. The different sugar modulation could be associated with the genetic diversity between the sugarcane cultivars which has been extensively studied and well reported [17, 19].

The individual saccharides estimations with greater precision in sugarcane juice by using high performance liquid chromatography (HPLC) has not extensively reported yet. The HPLC based saccharide analysis require very less sample and time as it coupled with the more sensitive detector. The detector such as diode array detector use for estimation of series of antioxidant components in consumable plant extracts [26] as well as HPLC-RI precisely detects trace alcohol impurities in organic solvents [35]. Albertson and Grof [1] measured the activity of key enzyme contributes for sucrose accumulation soluble phosphate synthase (SPS) and soluble acid invertase (SAI) in four high and four low commercial cane sugar (CCS) clones with HPLC-PAD system with HR-X Hamilton carbohydrate column. The polar extract of mature and immature leaf of sugarcane showed considerable variations in saccharides estimated by using gas chromatography [5]. Recently, the non enzymatic osmo-protectent ascorbic acid (vitamin C) determined in wheat plants and Arabidopsis leaf tissues by using HPLC-UV detector [32].

However, the trend in sugar variation among high and low sucrose sugarcane cultivars at the genetic level has been studied by using different DNA based molecular markers such as ISSR, SSR [19, 18, 10], TRAP, SNP [9]. The Random Amplified Polymorphic DNA (RAPD) markers has been use for molecular analysis of sugarcane due to its higher rate of polymorphism in complex sugarcane developed through inter and intra specific hybridization. RAPD based genetic analysis of tissue culture raised plants of Co86032 and CoC 671 which showed higher percentage of clonal fidelity and importance of sugarcane clones [8]. Similarly, the RAPD based clonal fidelity has reported in CoSe 01235 and CoSe 01424 sugarcane varieties [22].

This paper describes the development of HPLC-RI method for identification and quantification of different saccharides (monosaccharide and disaccharide) in sugarcane juice and correlation of individual sugars, with total soluble solids (Brix). However, the genetic evaluation of the same cultivars has studies by the different RAPD molecular markers.

Material and Methods

Plant material collection and extraction of juice

Total six sugarcane cultivars (Saccharum spp.), three high sucrose (HS) and three low sucrose (LS) sugarcane were grown in sugarcane research station, Pune, India situated at 18° 31' N latitude and 73° 55' E longitude. The agronomic practices were commencing to the sugarcane maturation phase. The stems and leaf samples were collected in triplicate from each cultivar at 300 days after planting (DAP), 360 DAP and 420 DAP in the morning time followed by storing in liquid nitrogen to stop sugar hydrolyzing enzymes and diurnal variation in enzyme activity.

The leaf material used for DNA isolation [2] and RAPD-PCR analysis, while stem material used for extraction of juice and estimation of brix, pol, purity and commercial cane sugar (CCS) by using AutoPol 880 saccharimeter (Rudolph Research analytica). The extracted juice filtered through 0.22 µm filters (laxbro) used for estimation of fructose, glucose, and sucrose on HPLC and it expressed in mg g-1 of fresh weight of tissue.

Sample preparation and HPLC analytical method

The sharp peaks of sugars were optimized with different mobile phase gradients. Furthermore, the samples were prepare
d in acetonitrile/water (75:25 v/v) as mobile phase and the reference standards of respective sugars were prepared in 100-300 ppm. For sugar analysis, Waters 515 HPLC pump equipped with isocratic system, rheodyne injector type (20µl loop volume) with flow rate 0.8 ml per minute, ultra amino column (150mm l, 4.6mm inside diameter, 3.0µm particle size and 100A° pore size) (Restek- USA) and Refractive Index (RI) detector controlled at 35°C was used. Chromatographic data were collected in Empower-2 software and other computer programs were use for correlation coefficient, regression analysis.

The obtained data were analyzed by one-way analysis of variance (ANOVA) using the statistical software SPSS 10.0, and the treatment means were compared by using Duncan's multiple range test (DMRT) at $P \leq 0.05$. Data were expressed as mean ± standard error (SE).

RAPD-PCR amplification and analysis

The RAPD-PCR amplification was performed on the isolated and quantified DNA (10 ng/µl) of respective cultivars (Table 1). Total ten RAPD primers were used for genetic evaluation study (Table 3). The PCR components and the amplification conditions were followed from the genetic analysis carried out in sugarcane with RAPD primers by Devarumath et al. [8]. The amplified product resolved on 1.5% (w/v) agarose gel in 0.5x TBE running buffer at constant voltage 75V and documented using gel documentation unit (UVITech).

Results and Discussion

The physiological and genetic variations has found significant in correlation with the sucrose and other saccharides accumulated during sugarcane development. The variations in individual saccharides of all cultivars at 300-420 DAP were estimated against the standard chromatogram generated by HPLC-RI (Fig. 1).

Saccharide analysis

The stem juice analysis during the maturation phase of HS and LS sugarcane accessions showed variable trend of mono and disaccharide concentrations in 300-420 DAP. The distinct sharp peaks of sugars were obtained in the acetonitrile/water (75:25%) mobile phase while, other gradients of mobile phase were found not suitable for analysis. This estimation of carbohydrates suggested the utility of high throughput HPLC technique for analysis of sugarcane juice within few minutes, against the minimum interference of reversible enzymatic activities. The utility of HPLC in identification of peaks with the similar retention time of individual sugars shows the robustness of technique, sample preparations and variation in run parameter by using sugarcane extracts. Moreover, the similar findings has mentioned in sugarcane (Saccharum spp. hybrid cv H50-7209) vacuole preparations with uridine diphosphate [14C] glucose which found the same retention time of disaccharide tentatively identified as laminaribiose [23] in acetonitrile/water (83:17 v/v) mobile phase. The isocratic system with RI detector used in present study minimizes the retention time less than 12 minutes. However, the isocratic system coupled with the pulsed ampherometric detector (PAD) used for determination of sugars compounds in olive plant extracts takes 15 minutes which found higher than our retention time [6]. This system could be used for the rapid detection of sugars. More or less, the similar HPLC-RI used for the sugar and sugar derivatives determination in chestnut and almond extracts [4], milk adulterations identification [29].

The Saccharide estimation in HS and LS showed variation in sugars. Among the HS cultivars, the average concentration of fructose and glucose were consistently reduced with respect to maturation of sugarcane however, sucrose dramatically increased from 300-360 DAP and decrease at 420 DAP (Fig. 2A, B and C). The increase in sucrose at 360 DAP supports the threshold of sucrose accumulation during maturation of sugarcane which found physiologically and biometrically important parameter for harvesting the sugarcane for maximizing the sucrose yield and recovery. However, in LS cultivars the rate of decrease in fructose and glucose was found slower as these

cultivars posses higher concentration of monosaccharides and lower concentration of sucrose found during 300-420 DAP. More interestingly, the CoVSI 48-188 showed higher fructose and glucose concentrations among the LS cultivars and it remain detectable in 420 DAP (Table 2 and Fig. 2). Conversely, the CoVSI 48-188 detected lowest sucrose concentration in all the time points in compared to the LS cultivars, this may be due to the less activity of sucrose synthesizing enzymes towards the sucrose formation [17]. This finding signifies the selection of consistent high and low sucrose sugarcane cultivars by using HPLC technique.

The average decrease in fructose and glucose at 300-420 DAP in HS and LS were found about 2.2 and 1.5 folds respectively however, sucrose modulation was found with an average of 12.2 folds from initial to the final time point. These changes in sugar concentrations confined to the time period of the sugarcane growth which can be utilized as a sugar based phytochemical marker. Fructose and glucose are found key monosaccharide which has involved in the sucrose synthesis and it modulated under different physiological changes in sugarcane studies [34]. The various physio-biochemical and environmental factors are responsible for the sucrose synthesis and accumulation which mainly affected by the key regulating enzymes, sucrose synthase (SS), sucrose phosphate synthase (SPS), soluble acid invertase (SAI) and neutral invertase (NI) associated with the synthesis and hydrolysis of sucrose. The individual enzyme activity and other parameters have significantly correlated among these selected cultivars [17]. Very few research reports on HPLC based sugar profiling have been reported in sugarcane however, the Grof et al. [15] analyzed the sugars in 4 high and 4 low CCS sugarcane genotypes and determined the SPS enzyme as a biochemical marker for high CCS hybrids selection. The HPLC based sugars analysis in different tissues of sugarcane has found important outcomes for the determination of drought-response genes expression during sucrose accumulation and water deficit condition in sugarcane culm [16]. Moreover, the sugar has significantly determined in the molasses using HPLC-RI detector [36]. The quantitative genetic of sugar accumulation (saccharides) estimated by HPLC-ELSD in pears of inter specific hybrids showed good relationship about active sucrose in pears [3]. This work was found more relational to the present study carried out in sugarcane.

The correlations of individual sugars with the brix were estimated by using Pearson correlation (r). The correlations analysis found to be strongly significant towards the modulation of sugars during maturation phase. The sucrose was positively correlated with brix and 'r' value estimated 0.92, 0.95 and 0.97 for 300, 360 and 420 DAP respectively (Fig. 2 a, b and c). However, the obtained monosaccharide, fructose 'r' value was found 0.74, 0.86, 0.86 and glucose 'r' value was found 0.70, 0.82 and 0.78 for 300, 360 and 420 DAP respectively (Fig. 2 a, b and c). This suggested the active synthesis of sucrose and its storage in stem by utilizing the precursor of simple sugars and their derivatives in mesophyll tissue of leaves [25]. The reason behind the variation in economically important sucrose could be associated with the enzymes which are predominating take parts in primary metabolism [18] and its localization in cellular compartments [14].

In future, the HPLC base analysis could be at the commercial level as sugarcane has rich source of carbohydrate, dietary fiber contents and antioxidant properties. These obtained results of sugar profiling on HPLC-RI also be helpful to uncover the more similar morphological and biometrical parameters between the cultivars, which are predominating found similar due to its high polyploidy level of genome size ~10,000 Mb [7]. Author has used these findings for the selection of diverse sugarcane genotypes for DNA fingerprinting study by using DNA based molecular markers as well as gene expression studies.

RAPD analysis for genetic variations

The genetic variability among the high and low sucrose accessions were analyzed by using RAPD-PCR molecular primers. Of the total 10 decamer RAPD screened (Table 2), the two

decamer primer gave intense banding pattern in the six sugarcane cultivars. The amplification product ranged from 0.1-1.5Kb. Some of the RAPD primers showed the polymorphic bands which differentiated the high and low sucrose sugarcane cultivars. From the total 10 primers analyzed, 40% of the primers (OPAB07, OPK07, OPK10 and OPK15) found polymorphic for the given locus. The polymorphic percentages were ranged from 17-40% obtained for OPK07 and OPK15 respectively. Total 61 alleles were amplified of which 8 alleles were found polymorphic with 13.11% (Table 3).

From the above results, it could be suggested that the genetically more similar species has less variation detected by the most polymorphic markers. The obtained 40% of polymorphic markers found less than the 90% polymorphic RAPD markers found in sugarcane diversity study [21] this could be due to the less number of RAPD markers and cultivars used in the present study. Due to higher polymorphic rate of selected primers, it could be use for the genetic discrimination of closely related sugarcane and economically important crops [8]. The VSI 434 and CoVSI 9805 showed polymorphic amplified band at 1.0Kb with OPAB07 primer (Fig. 3) which was found absent in all the low sucrose sugarcane cultivars, this polymorphism might be associated with the high sucrose accumulation in HS cultivars.

The results obtained through this experiment confirmed the efficiency of RAPD-PCR for determination of genetic relatedness among different cultivars. The carbohydrate variation obtained from the HPLC could helps to understand the genetic variability with other markers. The less genetic discrimination could not represent the genetic relatedness (dendrogram) between sugarcane cultivars. The RAPD markers have been extensively used in population genetics, biodiversity and studies of relationships among sugarcane species at different level [8, 13]. However, the non-specific amplification and robustness could reduce the reproducibility of markers [33]. Therefore, the application of more primers would be help to develop the major insights in sugarcane genomics study by utilizing the HPLC based phytochemical analysis.

Table 1. List of the three high sucrose (HS) and three low sucrose (LS) sugarcane cultivars used for the carbohydrate analysis and genetic evaluation

Sr. No	Sugarcane Cultivars	Parentage	Total soluble sugars (brix)[a]
1	VSI 434 (HS)	Somaclones of CoC671	18.3
2	Co VSI 9805 (HS)	Co8371PC	21.93
3	Co 85004 (HS)	Co6304×Co740	22.59
4	Co VSI 5-86 (LS)	Co87002×CoH70	14.3
5	CoVSI 48-188 (LS)	CoC8001GC	15.47
6	MS 68/47 (LS)	Co798×Co775	14.95

[a]Brix value was estimated at the 360 DAP preferably used for the cane crushing in sugarcane industry.

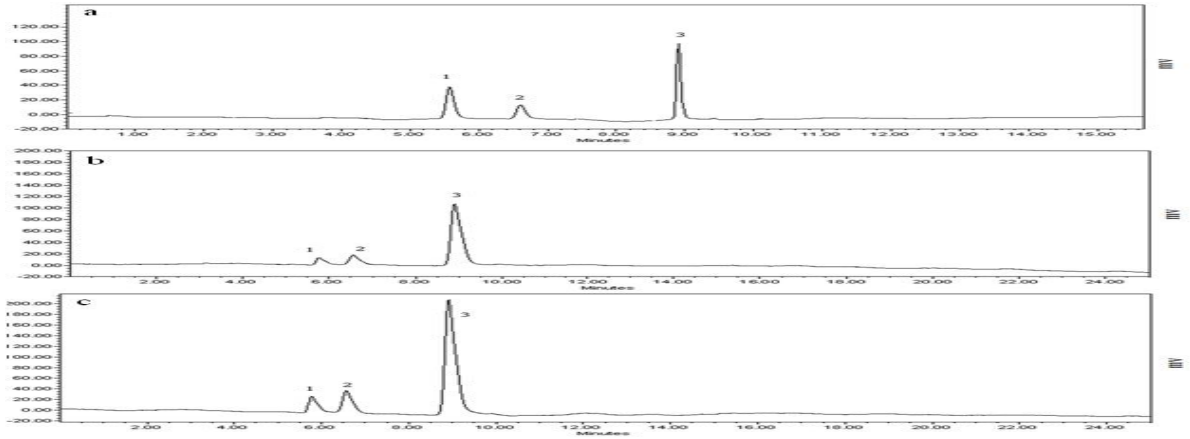

Fig.1. Chromatogram representing the isolated peaks of fructose (1), glucose (2) and sucrose (3) of standards (a), Co85004 (b) and CoVSI 48-188 (c) sugarcane cultivars.

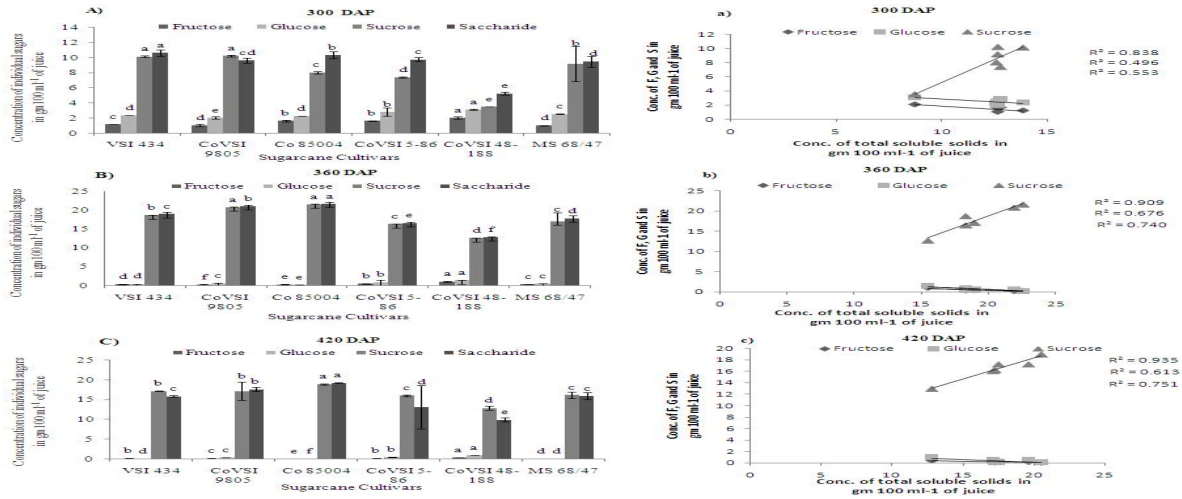

Fig.2. Concentrations of sugars (A, B and C) and its correlation with total soluble sugars (a, b and c) at 300-420 DAP in HS and LS sugarcane cultivars.

Table 2. Mean ± SE of fructose, glucose, sucrose, saccharides and brix estimated in individual HS and LS sugarcane cultivars in different development stages of 300 DAP-420 DAP. One way analysis with the Duncken test was performed for 5% significance ($p > 0.05$)

	Carbohydrate concentrations mg g^{-1}			Biometrically analyzed sugar and total soluble sugar (%)	
Cultivars	Fructose	Glucose	Sucrose	Saccharide	Brix
300 DAP					
VSI 434	1.20±0.02	2.36±0.006	10.15±0.13	10.64±0.42	13.87±0.19
CoVSI 9805	1.06±0.17	2.03±0.16	10.24±0.12	9.62±0.33	12.68±0.18
Co 85004	1.63±0.09	2.28±0.008	8.05±0.15	10.35±0.45	12.61±0.61
CoVSI 5-86	1.64±0.05	2.84±0.52	7.40±0.04	9.77±0.24	12.8±0.39
CoVSI 48-188	2.05±0.15	3.12±0.07	3.53±0.02	5.26±0.18	8.75±0.07
MS 68/47	1.01±0.05	2.54±0.02	9.20±2.33	9.47±0.72	12.67±1.54
360 DAP					
VSI 434	0.36±0.01	0.48±0.01	18.66±1.46	19.02±0.11	18.30±0.02
CoVSI 9805	0.20±0.01	0.62±0.009	20.76±0.22	21.05±0.93	21.92±1.65
Co 85004	0.29±0.14	0.23±0.02	21.54±1.25	21.68±0.74	22.59±0.18
CoVSI 5-86	0.50±0.02	0.95±0.07	16.39±1.83	16.63±0.74	18.30±0.15
CoVSI 48-188	1.08±0.11	1.48±0.02	12.60±1.11	12.83±0.50	15.47±0.33
MS 68/47	0.41±0.03	0.58±0.27	17.02±1.59	17.80±1.27	18.95±0.91
420 DAP					
VSI 434	0.21±0.02	0.12±0.01	17.22±0.02	15.88±0.20	17.50±0.16
CoVSI 9805	0.16±0.12	0.41±0.01	17.22±2.24	17.67±0.49	19.61±0.37
Co 85004	0.02±0.01	0.08±0.009	18.95±0.16	19.26±0.04	20.51±0.01
CoVSI 5-86	0.20±0.009	0.45±0.06	16.05±0.17	13.12±5.50	17.12±5.18
CoVSI 48-188	0.43±0.01	0.97±0.006	12.92±0.55	9.92±0.53	12.83±1.33
MS 68/47	0.06±0.006	0.11±0.06	16.22±0.81	15.98±0.87	17.23±0.28

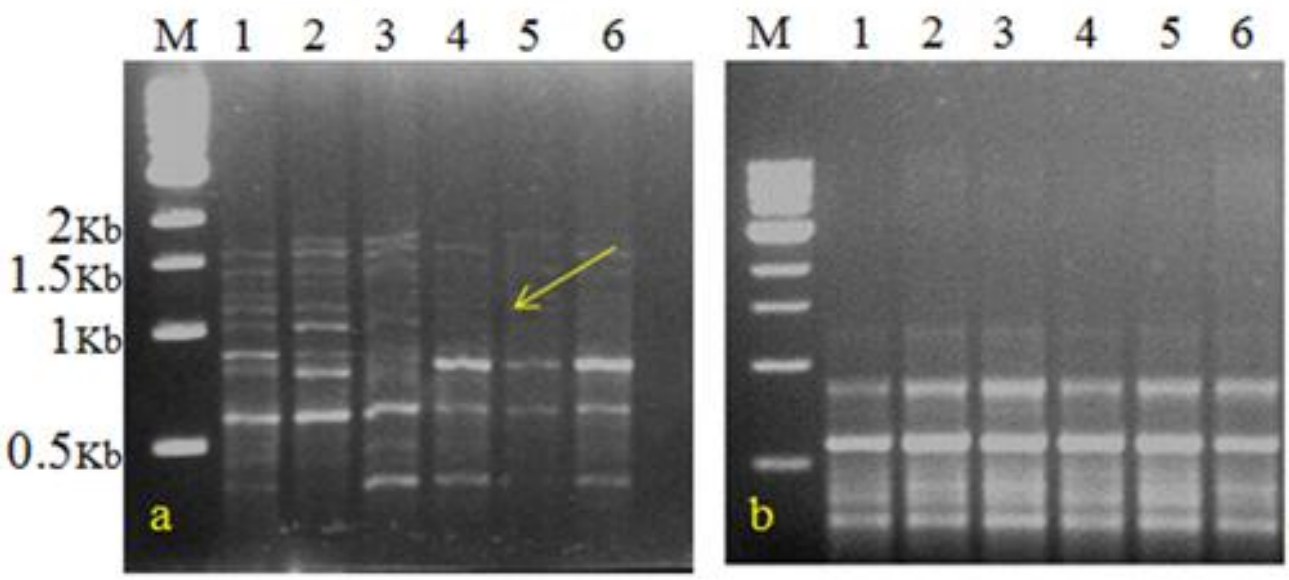

Fig.3. Genetic profiling of six sugarcane cultivars by using RAPD markers OPAB07 (a), OPAB02 (b) showed polymorphic and monomorphic bands respectively. M- 10Kb DNA ladder and lanes1-6 represent cultivar names mentioned in table 1 (Arrow indicating absence of amplicon).

Table 3. List of the decamer RAPD markers analyzed on selected sugarcane cultivars.

Sr. No.	Primer Name	Primer Sequence 5' 3'	Band Observed	Polymorphic Bands	Percentage Polymorphism (%)
1	OPAB02	TGATCCCTGG	5	0	0
2	OPAB07	GTAAACCGCC	9	3	33
3	OPH01	GGTCGGAGAA	5	0	0
4	OPH06	ACGCATCGCA	6	0	0
5	OPH08	GAAACACCCC	7	0	0
6	OPK07	AGCGAGCAAG	6	1	17
7	OPK10	GTGCAACGTG	7	2	29
8	OPK12	TGGCCCTCAC	5	0	0
9	OPK15	CTCCTGCCAA	5	2	40
10	OPK20	GTGTCGCGAG	6	0	0
Total			61	8	
Average			6.1	0.8	11.9

Conclusions

The HPLC-RI found more simple, accurate and reliable method for qualitative and quantitative analysis of carbohydrates in sugarcane stalk juice with distinct peak resolution in less than 12 minutes. This practical approach would be helpful to study the different plant extracts under solvent dilutions. Positive correlation at linearity was observed for sucrose while, the negative correlations at linearity were observed for fructose and glucose against total soluble solids suggested the active accumulation of sucrose at 360 DAP

which found suitable for maximum sugar recovery at industrial level. However, the genetic variations attributed by the OPAB07 primers could be utilized towards the development of sequence characterized amplified region (SCAR) molecular markers. This might be associated to the high sucrose content as well as it could be for discrimination of high yielding sugarcane varieties develop through sugarcane breeding programs.

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