HPLC profiling of flatulence and non-flatulence saccharides in eleven ricebean (*Vigna umbellata*) varieties from North-East India

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Revised: 16 February 2019 / Accepted: 21 February 2019 / Published online: 1 March 2019
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Abstract HPLC method was optimized for analysis eight flatulence and non-flatulence saccharides in 11 ricebean varieties using Spherisorb NH₂ chromatographic column within 20 min by isocratic mobile phase containing acetonitrile and water in ratio of 70:30 at flow rate of 1 mL/min. The glucose, sucrose, raffinose, stachyose, verbascose and ajugose content in these varieties ranged from 157.63–365.08, 202.61–997.74, 196.29–429.5, 692.7–1480.67, 47.66–130.36 and 0.27–8.82 mg/100 g, respectively. The total flatulence saccharides (FS) were range between 937.13 ± 44.31 (JCR-08-12) and 1944.55 ± 83.57 (JCR-08-32) mg/100 g. Among the total FS, the content of stachyose was found highest in all varieties than other FS. Principal component analysis (PCA) revealed that glucose, sucrose, raffinose, stachyose, verbascose, ajugose, and total FS contributed 50.03% variation among the varieties. The eleven varieties were classified into three groups based on saccharides content by PCA. The group-A (JCR-08-12) and group-B (JCR-08-7, JCR-08-16, JCR-50, JCR-13-11, JCR-13-13 and *Nagadal*) had very low and low content of total FS, respectively.

Keywords Flatulence · Raffinose · Oligosaccharides · HPLC · Ricebean · Xylose · Ajugose

Introduction

Biochemically, ‘saccharide’ is a structural unit of carbohydrates which builds mono-saccharide, disaccharide, oligosaccharide and polysaccharide by blocking its one, two, few and multiple unit into these structure, respectively. The mono-saccharides such as glucose, galactose, fructose, maltose has significant nutritional contribution and act as a primary source of energy. The non-starch polysaccharides are hydrolyzed into arabinose and xylose by microbiota of large intestine which can protect myocardial injury (Lim et al. 2016). Hence, glucose, galactose, fructose, maltose, xylose, and arabinose are considered as non-flatulence saccharides (NFS).

The raffinose, stachyose, verbascose, ajugose, and unnamed so far longer-chain oligosaccharides up to non-saccharide are called raffinose family oligosaccharides (RFOs) or α -galactosides which act as desiccation protectant in stored seeds (Cerning-Beroard and Filiatre-Verel 1980; Sengupta et al. 2015). RFOs are considered as an anti-nutrient by the nutritionists because of flatulence activity which results in stomach rumblings, abdominal discomfort, pain, cramps and diarrhea (Andersen et al. 2005). Human cannot digest RFOs due to lack of α -galactosidase enzyme in digestive system. RFOs accumulate in lower intestine and undergo anaerobic fermentation process by gut microbiota which release the flatulence gases such as H₂, CO₂, and traces of CH₄ (Kannan et al. 2017). Therefore, these saccharides are also called flatulence saccharides (FS). Apart from flatulence activity, the scientific reports also stated that FS have many health benefits such as mineral absorbability, anti-cardiovascular, anti-carcinogenic, and anti-diabetic activity (Masao 2002; Tajoddin et al. 2012).

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Ricebean (*Vigna umbellata*), one of the promising bean popularly used in South-East Asia has 52.23–68.5% of carbohydrate which can be fractioned into starch, dietary fibre, NFS and FS (Bepary et al. 2016).

Saccharides has been analysed by using different chromatographic techniques under different types of detection. Profiling of saccharides in HPLC with refractive index (RI) detector using spherisorb NH₂ column and acetonitrile–water as the mobile phase, is being considered popular, simple, cost effective method (Tihomirova et al. 2016). These analytical conditions has been used for analysis of mono, disaccharides in infant formulae (Ferreira and Ferreira 1997), oligosaccharides in leguminous seeds (Knudsen 1986), and mono, oligosaccharides in vegetables (Hernandez et al. 1998) etc. These exiting methods were unable to analyze the FS and NFS simultaneously under single injection and had weak peak resolution. Thus, this study was planned to overcome the exiting problems associated with exiting HPLC methods and also to improve the analytical efficiency.

Although information on NFS and FS are available in ricebean, studies on ricebean variety from North-East India are scanty. Moreover, earlier quantification of NFS as well FS was based on conventional chemical analysis. The present study carried out the HPLC analysis of xylose, glucose, sucrose, maltose, raffinose, stachyose, verbascose and ajugose in selected ricebean varieties.

Materials and methods

Sample collection

The ten authentic North-Eastern ricebean varieties namely JCR-08-7, JCR-08-8, JCR-08-10, JCR-08-12, JCR-08-15, JCR-08-16, JCR-08-32, JCR-13-11, JCR-13-13, and JCR-50 were obtained from the AICRP on Forage Crops and Utilization, Assam Agricultural University, Jorhat, India whereas eleventh variety e.i., *Nagadal* was purchased from local market of Dimapur, Nagaland. The germplasm of JCR series varieties were collected from tribal areas of North-Eastern states and used for breeding purpose. These varieties are differed in certain botanical as well as agro-nomical characteristics (Hollington et al. 2009). The samples were ground by using vibratory micro mill (Pulverisette, M/S. Fritsch, Germany) using 250 µm sieve.

Chemicals

D(+)xylose, D(+) glucose, sucrose and maltose monohydrate standard were purchased from M/S SD Fine Chemicals (Mumbai, India) and raffinose, stachyose were from M/S Sisco Research laboratory (Mumbai, India) while

verbascose, analytical-grade ethanol and HPLC-grade acetonitrile from M/S Sigma (Madrid, Spain). As ajugose is not available, it was extracted from black gram as per the procedure mentioned by Kotiguda et al. (2006). The purity of ajugose is confirmed by HPLC.

HPLC and chromatographic condition

HPLC profile of eight saccharides were analyzed as per the procedure described by Hernandez et al. (1998) with modification. The chromatographic analysis was carried out by using HPLC system (M/s. JASCO Instruments, Hachioji-shi, Tokyo, Japan) which has an online degasser 4-channel(DG2080-54), dual pump (PU-2080), a solvent mixer (Mx-208031), a RI detector (RI-2031plus) and a manual injector of 20 µL sample loop (Rheodyne, Cotati, CA, USA) linked through HPLC Net connector(LC-Net-11/ADC 474) to desktop. Chromatographic separations were carried out in a Spherisorb NH₂ (250 × 4.6-mm column with 5 µm) column with guard column by using isocratic mobile phase acetonitrile–water mixture. The injection volume was 0.02 mL. The total run time was 20 min. Data were collected and analyzed with the Borwin software (version 1.5, 2001).

Preparation standard mixtures and calibration curves

All the saccharides standards (2.1–32.2 mg) were dissolved individually in 5 ml of HPLC grade water to get stock solution. The stock solutions were filtered through 0.2 µm pore size syringe filter and stored at – 20 °C until further usage. Individual saccharides peaks were calibrated against their working standard ranged between 0.01 and 0.9 mg/mL per injection. The calibration standards mixes were prepared with aliquot taken from standard stock solution of each saccharide in the desired concentration (Table 1).

Optimization and validation of method

In order to, get better peak resolution and also simultaneous analysis of NFS and FS, different combinations of mobile phase [acetonitrile and water mixed in the proportion of 50:50, 60:40, 70:30, 80:20] in isocratic mode were studied at different flow rates (0.5–1.2 mL/min.). Different saccharides (individual as well as mix standards) were injected to find out the retention times at optimized instrumental conditions. For any quantitative study, optimization and validation of analytical method is essential requirements; if some modifications are being made both in terms of method's process as well as analytical environments. The obtained optimized method was validated as per the guidance note from International Conference on

Table 1 Method validation parameters

Standard	Standard range (mg/mL)	Calibration curve	Correlation coefficient (r^2)	LOD (mg/mL)	LOQ (mg/mL)	RSD _P (%)	RSD _R (%)	Recovery (%)	RSD _A (%)
Xylose	0.1010–0.569	$Y = 41,898x - 116.7$	0.991	0.0315	0.0954	2.34	1.78	95.23	2.33
Glucose	0.130–0.948	$Y = 34,245x + 43.24$	0.998	0.0310	0.123	0.96	1.85	98.44	0.95
Sucrose	0.205–0.534	$Y = 49,735x + 15.01$	0.997	0.051	0.155	0.98	1.52	97.12	0.98
Maltose	0.170–0.456	$Y = 44,547x - 330.5$	0.993	0.0408	0.144	1.22	1.78	95.78	1.34
Raffinose	0.160–0.630	$Y = 63,013x - 119.8$	0.998	0.046	0.140	2.1	1.43	94.86	1.45
Stachyose	0.320–0.640	$Y = 34,798x - 561.9$	0.987	0.102	0.310	2.34	1.53	93.76	2.11
Verbascose	0.577–0.976	$Y = 13,032x - 108.6$	0.998	0.191	0.577	2.86	1.78	94.42	2.33
Ajugose	0.610–1.056	$Y = 21,456x - 210$	0.975	0.230	0.589	3.10	2.95	92.87	2.88

LOD limit of detection, LOQ limit of quantification, RSD_A relative standard deviation of recovery assay, RSD_P method precision (n = 6), RSD_R method repeatability (n = 3)

Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [ICH Topic Q2 (R1) 2005]. As per the ICH guidance note, linearity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy are key parameters for validation of any analytical methods, hence these parameters were studied to validate the optimized method. The selectivity of a chromatographic method was judged based on the resolution of analyte peak in the baseline which is uniquely separated from other analytes in sample. To study the linearity of the method, the calibration standard mix containing eight saccharides at four concentrations was prepared and the range of standard calibration mix is depicted in Table 1. Each concentration of the mixed standard solution was injected in triplicate. The calibration curves were constructed by plotting the peak areas versus concentrations of each saccharide. LOD and LOQ of the method were calculated as 3.3X (SD/S) and 10X (SD/S) respectively, where 'SD' is the standard deviation of the response and 'S' is the slope of the regression line. Within a day and day-to-day variations were determined by running standard mix in triplicates for three different days. Percentage of relative standard deviation was used as a measure of precision. Accuracy was estimated by means of recovery assays. For evaluation of recovery, six samples of ricebean were spiked with known concentrations of saccharides prior to extraction and quantification.

Sample preparation

Ricebean flour (5 g) was added to an Erlenmeyer flask (250 mL capacity) containing 50 mL of 70% ethanol (v/v) and placed on an orbital shaker at 130 rpm for 12 h. The contents of the flask were filtered through Whatman No. 1 filter paper and the residues were further washed with 25 mL of 70% ethanol. The combined filtrates were

evaporated in a rotary vacuum evaporator at 60 °C. The concentrated sugar syrup obtained from vacuum evaporation was dissolved in 10 mL of distilled water (Mulimani and Devendra 1998). The 10 mL samples were centrifuge at 10,000 rpm for 20 min and then filtered through a 0.45-µm Millipore membrane (Millipore), and 0.02 mL of filtered samples were injected into the chromatographic system (Sánchez-Mata et al. 2002). The identification and quantification of each saccharides present in ricebean samples were estimated by comparing retention time and peak area between a standard and a sample (Fig. 1).

Statistical analysis

The statistical analysis of results carried out using MS-Office analysis tool-pack for ANOVA and correlation coefficient, Duncan's test. The principal components analyses (PCA) were carried out by using statistiXL software (Trial version). For PCA, saccharides data was analyzed by setting Eigen value more than zero. Proper saccharides data distribution in scatter plot was carried out by correlation as model matrix and varimax as rotational method.

Results and discussion

Optimization and method validation

The isocratic mobile phase [70% acetonitrile: 30% water (v/v)] and flow rate of 1 mL/min was showing good peak resolution of all saccharides simultaneously, hence this was considered as optimum condition. The each saccharide was identified based on their retention time at optimized chromatographic conditions (Fig. 1). The method validation parameters such as calibration curve, LOD, LOQ, precision

Fig. 1 Chromatogram of saccharides in standard and sample

and accuracy are presented in Table 1. The calibration curves for each saccharide were depicted excellent linearity in the experimental range with a correlation coefficient $r^2 > 0.975$. The LOD of the method ranged between 0.0310 mg/mL (glucose) to 0.230 mg/mL (ajugose)

whereas LOQ were ranged between 0.0954 mg/mL (xylose) to 0.589 mg/mL (ajugose). The intra-day precision (expressed as percentage relative standard deviation: % RSD_p) was found below 3.10% for all saccharides. However, interday precision (expressed as percentage relative

standard deviation: % RSD_R) was fall below 1.78% except ajugose which showed good repeatability of the methods. Percentage of recovery was ranged from 92.87% (ajugose) to 98.44% (glucose) which testify the method accuracy.

Determination saccharides profile in ricebean

The content of NFS and FS were estimated with validated HPLC method in eleven ricebean varieties (Table 2). Among the studied NFS, xylose and maltose were not detected in eleven ricebean varieties tested in present study. Only simple extraction with 70% ethanol might not be sufficient to extract detectable xylose. This may be the probable reason for non-detection of xylose in present study. Longe (1980) studied xylose content in cell wall constituents of cowpea after discarding 80% ethanolic extracts. In that study, xylose was extracted by treating cell wall constituents with sulphuric acid to get cell wall hydrolysate and then hydrolysate was neutralized with barium carbonate and used for xylose analysis. Maltose was also not detected in native bean such as kidney bean, lima bean, African black bean, yam bean (Phillips 1989). The content of maltose in grain depends on enzymatic activities of grain, exogenous phosphate content and condition during storage. Lack of enzymatic activities, absence of exogenous phosphate content in grain and cold stress while storing can prevent the starch breakdown which ultimately reduce maltose accumulation in grain (Lu and Sharkey, 2006). These may be the reason for non-detection of maltose in ricebean. The total NFS (glucose and sucrose) were ranged (mg/100 g) from 360.24 ± 20.22 (JCR-08-12) to 1307.93 ± 51.43 (JCR-13-11). The average content

(mg/100 g) of glucose and sucrose in ricebean were 256.41 ± 72, and 653.86 ± 214, respectively. Kaur and Kapoor (1992) reported reducing sugar in the range 278–359 mg/100 g in five ricebean varieties. The content of glucose varied between 157.63 ± 8.43 mg/100 g (JCR-08-12) to 365.08 ± 15.32 mg/100 g (JCR-50). The highest content of sucrose was found in the variety JCR-13-11 (997.74 ± 31.21 mg/100 g) and lowest in variety JCR-08-12 (202.61 ± 11.98 mg/100 g). Sucrose content in the varieties namely JCR-08-8, JCR-08-15 and JCR-08-16 were not varied significantly ($p < 0.05$).

The average value of FS (mg/100 g) in eleven ricebean varieties were as follows-raffinose: 305.19 ± 62, stachyose: 1131.17 ± 221, verbascose: 90.46 ± 24, ajugose: 4.33 ± 3.58 and total FS: 1531.12 ± 286. The average value of raffinose, verbascose, and ajugose were found lower than the reported value whereas average value of stachyose was compliance with reported value (Malhotra et al. 1988; Girigowda et al. 2005; Katoch 2013). Malhotra et al. (1988) studied raffinose, stachyose, verbascose content (mg/100 g) in 14 ricebean varieties and value reported 320–910 raffinose, 950–1980 stachyose, 1400–1880 verbascose. Girigowda et al. (2005) studied ajugose content in 12 cultivars of black gram and reported 830–1650 mg/100 g. The average content total FS in 11 ricebean varieties were exhibited lower than the value (3280–5870 mg/100 g) reported by Katoch (2013). Among the FS, the content of stachyose was found highest in all the varieties of ricebean than other FS. The lower value of FS content in ricebean varieties may be due to genetic factors, environmental factors, stage of harvesting, and enzymatic activities in stored grain. The raffinose content (mg/100 g) in

Table 2 Flatulence and non-flatulence saccharides content (mg/100 g) in eleven ricebean varieties

Non-flatulence saccharides				Flatulence saccharides (FS)			
Variety	Glucose	Sucrose	Raffinose	Stachyose	Verbascope	Ajugose	Total FS
JCR-08-7	179.01 ± 13.44 ^a	540.94 ± 23.12	338.61 ± 15.21	1037.5 ± 56.11 ^a	70.83 ± 4.33 ^a	1.19 ± 0.54 ^{bc}	1448.13 ± 76.19
JCR-08-8	301.94 ± 16.11 ^d	724.92 ± 25.21 ^a	429.5 ± 13.20	1288.26 ± 50.32 ^b	80.54 ± 5.11 ^b	3.94 ± 0.87 ^d	1802.23 ± 69.5 ^b
JCR-08-10	186.76 ± 15.22 ^a	586.63 ± 26.36	320.75 ± 10.33 ^a	1277.91 ± 65.11 ^b	68.96 ± 4.32 ^a	4.42 ± 0.79 ^d	1672.04 ± 80.55
JCR-08-12	157.63 ± 8.24	202.61 ± 11.98	196.29 ± 09.11	692.7 ± 32.11	47.66 ± 3.06	0.49 ± 0.03 ^{ab}	937.13 ± 44.31
JCR-08-15	234.77 ± 12.42 ^b	725.84 ± 28.52 ^a	320.62 ± 17.32 ^a	1376.83 ± 67.32	106.75 ± 5.21 ^c	8.82 ± 0.67	1813.02 ± 90.52 ^b
JCR-08-16	269.64 ± 11.45	742.6 ± 25.72 ^a	309.68 ± 12.01	975.43 ± 57.11	86.14 ± 6.93	4.17 ± 0.66 ^d	1375.42 ± 76.11
JCR-08-32	238.04 ± 15.31 ^b	605.3 ± 23.57	331.78 ± 14.93	1480.67 ± 60.87	130.36 ± 7.22	1.74 ± 0.55 ^c	1944.55 ± 83.57
JCR-50	365.08 ± 19.81 ^c	499.89 ± 19.22	324.3 ± 17.75 ^a	1080.16 ± 65.11 ^a	98.22 ± 6.32	7.45 ± 0.75	1509.91 ± 89.93 ^a
JCR-13-11	310.19 ± 20.22 ^d	997.74 ± 31.21	295.97 ± 11.30	1174.43 ± 59.28	117.71 ± 9.11	11.42 ± 1.05	1599.53 ± 80.74
JCR-13-13	364.94 ± 17.92 ^c	930.05 ± 30.43	216.51 ± 10.32	947.45 ± 47.54	78.24 ± 5.27 ^b	0.27 ± 0.02 ^a	1242.47 ± 63.15
Nagadal	212.56 ± 10.32	635.99 ± 27.28	273.07 ± 14.32	1111.56 ± 55.54 ^a	109.61 ± 8.37 ^c	3.68 ± 0.44 ^d	1497.91 ± 78.67 ^a

Each value is average of triplicate determination ± SD. Mean values in a column sharing a common letter are not statistically different ($p < 0.05$)

Fig. 2 Factor analysis-scatterplot of ricebean varieties grouped based on saccharides content (PC principal component)

ricebean varieties fluctuate between 196.29 ± 09.11 (JCR-08-12) and 429.5 ± 13.20 (JCR-08-8) and varied significantly ($p > 0.05$) among the varieties except JCR-08-10, JCR-08-15, JCR-50. The lowest content of stachyose was ascertained in variety JCR-08-12 (692.7 ± 32.11 mg/100 g) whereas highest content was found in JCR-08-32 (1480.67 ± 60.87 mg/100 g). The range of verbascose and ajugose content (mg/100 g) were found 47.66–130.36 and 0.27–8.82, respectively. JCR-08-32 had the highest content of total FS (1944.55 ± 83.57 mg/100 g) whereas lowest content found in JCR-08-12 (937.13 ± 44.13 mg/100 g). The flatulence saccharides in 11 ricebean varieties were found lower than the reported values for other pulses. Oomah et al. (2011) reported flatulence saccharides in different pulses as 0.1–2.6% of raffinose, 0.1–5.5% of stachyose, 0.04–3.8% verbascose and 0.06–2.0% ajugose. The current study observed that ricebean varieties had low level of total FS which may fit to promote the growth of probiotic microbiota in human digestive system. Hence, this could be used as prebiotic food ingredients in the development of functional foods (Hayakawa et al. 1990).

The principal components (PC) analysis was carried out to find principal factors responsible for variability in

ricebean varieties. PC1, PC2, PC3, PC4, PC5 contribute all together 98.11% variation of studied variables (saccharides). A substantial variation (50.03%) was shown by PC1 with loading factors that ranged 0.385–0.921 which were contributed by all saccharides. The loading factor of PC2 (variation 21.67%) was varied from -0.760 to 0.429 was positively contributed by raffinose, stachyose and total FS whereas negatively by glucose, sucrose, verbascose and ajugose. The PC3 (variation 12.61%) had loading factors -0.330 to 0.658 which were positively contributed by stachyose, verbascose, ajugose and negatively by glucose, sucrose, raffinose and total FS. The cumulative variation contributed by PC1 and PC2 was 71.69% which was the maximum among the PCs combined variations. Hence, two factors had been selected to group the varieties. Based on scatter plot developed from varimax rotation method, the ricebean varieties were divided into three groups (A, B and C) on the basis of saccharides content (Fig. 2). The first group or group-A (PC1: -2 to -1.5 ; PC2: 0 to -0.5) contain only JCR-08-12 with lowest content of total FS and total NFS (glucose and sucrose). This variety can be used in breeding purpose to develop variety with low FS content. Second group or group-B (PC1: -1.0 to 0 ; PC2: -1

to 1.5) contain JCR-08-7, JCR-08-16, JCR-50, JCR-13-11, JCR-13-13 and *Nagadal* with low total FS content. The third group or group-C (PC1: 0.5–1.5; PC2: – 2 to 2) contains only JCR-08-8, JCR-08-10, JCR-08-15, JCR-08-32 which had highest amount total FS. The varietal variation in terms saccharides content is clearly noticeable from component analysis. The varieties which belong to same group may be having same genotypical as well as phenotypical characteristics.

Conclusion

The modified HPLC method for determination of flatulence and non-flatulence saccharides was optimized and validated. The content of saccharides in ricebean varieties showed greater variation which was 50.03% of total varietal variation. Ricebean varieties were low in flatulence saccharides content compared to other pulses. The average content of glucose, sucrose, raffinose, stachyose, verbascose and ajugose in 11 ricebean varieties was 256.41 ± 72 , 653.86 ± 214 , 305.19 ± 62 , 1131.17 ± 221 , 90.46 ± 24 and 4.33 ± 3.58 mg/100 g, respectively while the xylose and maltose were not detected in this study. The total non-flatulence saccharides (glucose and sucrose) were 910.28 ± 268 mg/100 g and total flatulence saccharides (FS) were 1531.12 ± 286 mg/100 g. The 11 ricebean varieties were falling under into 3 categories such as group-A (JCR-08-12), group-B (JCR-08-7, JCR-08-16, JCR-50, JCR-13-11, JCR-13-13 and *Nagadal*) and group-C. Group-B ricebean varieties had lower total FS and may be suitable for individual with flatulence issues. The varieties with low FS content may be used as prebiotic food ingredient for the development of functional foods.

Acknowledgements The authors acknowledge the support of the Ministry of Minority Affairs, Govt. of India, New Delhi, and University Grant Commission, New Delhi, by providing Fellowship to carry out the research works. The authors acknowledge the Director, DFRL, Mysore, for providing facilities, support and encouragement to carry out this research and AICRP on Forage Crops, Department of Plant Breeding and Genetics, Assam Agricultural University, Jorhat for providing the rice-bean varieties for study.

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