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ScienceDirect
Food Chemistry 106 (2008) 324–330
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Comparison of two HPLC systems and an enzymatic method for quantification of soybean sugars

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Received 20 September 2006; received in revised form 24 April 2007; accepted 29 April 2007

Abstract

Successful breeding programs need fast and reliable methods for analyzing sugar composition in new soybean (*Glycine max* (L.) Merrill) lines. The efficiency to quantify the major sugars, including glucose, fructose, sucrose, raffinose, and stachyose, in five soybean lines with two HPLC systems and an enzymatic procedure were compared. Soluble sugars in soybean were extracted with water at a solvent-to-sample ratio of 5:1 at 50 °C for 15 min, and analyzed by high-performance size exclusion chromatography with refractive index detection (HPSEC-RI), high-performance anion-exchange chromatography with pulsed-amperometric detection (HPAEC-PAD), and a raffinose-series oligosaccharides assay procedure. All three methods produced comparable and reproducible results. The HPAEC-PAD method was more sensitive, faster and capable of separating all five major sugars in soybean with improved peak resolution compared with the HPSEC-RI method, and is recommended for soybean breeding programs. The enzymatic procedure required no expensive instrumentation and less sample preparation, but could not quantify individual raffinose and stachyose.

Keywords: Soybean sugars; HPSEC-RI; HPAEC-PAD; Raffinose; Stachyose

1. Introduction

The amount of total soluble sugars in soybean seeds varies among varieties, ranging from 6.2% to 16.6%, and sucrose, raffinose, and stachyose comprise almost 99% of the soybean soluble sugars (Kawamura, 1967). Sucrose is the most abundant sugar in soybean ranging from 3% to 10% and responsible for enhancing the sweet taste of soyfoods (Taira, 1990), whereas stachyose (0.6–5.8%) and raffinose (0.1–1.8%) (Hymowitz & Collins, 1974; Trugo, Farah, & Cabral, 1995) are not digestible. One way to improve the sugar composition of soybean and thereafter its marketability as food and feed is by breeding. For a successful breeding program, breeders need efficient and reliable methods to analyze sugar composition in new soybean lines.

Several methods have been reported for the determination of sugars in soybean and other legumes. The colorimetric method by Dubois, Gilles, Hamilton, Rebers, and Smith (1956) gives a reliable, but only the total sugar content. Paper chromatography (Lineback & Ke, 1975; Pazur, Shadaksharaswamy, & Meidell, 1962; Shallenberger & Moores, 1957) and thin layer chromatography (Tanaka, Thananunkul, Lee, & Chichester, 1975) provide qualitative analysis, but the results are difficult to quantify. Gas chromatography is very sensitive; however, it is laborious due to the need of sugar derivatization (Aman, 1979; Delente & Ladenburg, 1972; Folkes, 1985; Molnar-Perl, Pinter-Szakacs, Kovago, & Petroczy, 1984). High-performance liquid chromatography (HPLC) has become the preferred method because of its simple and efficient separation and quantification of sugars (Black & Bagley, 1978; Ladish & Tsao, 1978; Rabel, Caputo, & Edward, 1976; Reyes, Wrolstad, & Cornwell, 1982). HPLC coupled with refractive index (RI) detection is commonly used in soybean

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doi:10.1016/j.foodchem.2007.04.065

and other plants for sugar analysis (Black & Bagley, 1978; Frias, Hedley, & Price, 1994; Johansen, Glitso, & Knudsen, 1996; Kim, Kim, & Hwang, 2003; Knudsen, 1986). RI detection offers a wide linear range for sugar analysis, but is not very sensitive for low concentrations (Lis & Sharon, 1978; Martens & Frankenberger, 1990). More recently, high-performance anion exchange chromatography coupled with pulsed-amperometric detection (HPAEC-PAD) becomes increasingly popular and has been extensively employed for sugar analysis (Cataldi, Campa, Angelotti, & Bufo, 1999; Cataldi, Margiotta, Lasi, & Di Chio, 2000; Frias et al., 1999; Mohamed & Rayas-Duarte, 1995; Rocklin & Pohl, 1983; Townsend, Hardy, Hindsgaul, & Lee, 1988). PAD is highly selective and sensitive because only reactive compounds will give response and at very low concentrations.

Enzymatic analysis has also been routinely used for sugar analysis due to the specificity and sensitivity of enzymes. Maughan, Saghai Maroof, and Buss (2000) used invertase and hexokinase to quantify the sucrose content in 149 soybean varieties. However, little work has been reported to compare HPLC and enzymes for soybean sugar analysis. The objective of this study was to compare three methods, HPAEC-PAD, HPSEC-RI, and enzymes, for quantifying soybean sugars. Their advantages and disadvantages with respect to sample preparation, and sugar quantification and identification were addressed.

2. Materials and methods

2.1. Materials

Seeds of five soybean lines, namely Hutcheson, Camp, SS-516, MFL-552, and 03CB-14, grown in Fayetteville, Arkansas in 2002 were provided by the Department of Crop, Soil and Environmental Sciences at the University of Arkansas, Fayetteville, AR. Fifteen grams of each variety were ground in a mill for 20 s (Knifetec 1095, Foss, Hoganas, Sweden) and the ground meal was screened through a 150-μm sieve (W.S Tyler, Mentor, OH) and used for sugar extraction. The moisture content of each ground meal was determined according to Approved Method 44-31 (AACC, 2000). Glucose, fructose, melibiose, sucrose, raffinose, stachyose, and maltoheptaose were obtained from Sigma Chemicals Co. (St. Louis, MO). A raffinose-series oligosaccharides enzymatic assay procedure (RSO 8/98) was purchased from Megazyme (Megazyme Intl Ireland Ltd, Wicklow, Ireland). All other chemicals were ACS grades.

2.2. Extraction and purification of soluble sugars in soybean meal

Soluble sugars were extracted by the procedure previously optimized (Giannoccaro, Wang, & Chen, 2006). One gram sample spiked with an internal standard, which was used to check recovery and to assure an accurate quantification of sugars (Black & Bagley, 1978; Li, Schuhmann, & Wolf, 1985), and 5 mL of distilled water were placed in a 50-mL centrifuge tube. Melibiose was used as the internal standard for a HPAEC-PAD system, while maltoheptaose was used as the internal standard for a high-performance size exclusion chromatography with RI detection (HPSEC-RI) system. Different standards were chosen for the efficiency of each individual analysis. The tube was capped, placed horizontally, completely immersed in a water bath at 50 °C, and shaken at 200 rpm for 15 min. After the extraction, the tube was centrifuged at 20,000*g* for 10 min and 2 mL of the supernatant were pipetted into another centrifuge tube.

The same extract purification procedure was applied to both systems, which followed the method of Black and Glover (1980) with modifications. Three millilitres of acetonitrile was slowly added into the centrifuge tube containing the supernatant with constant shaking to precipitate the residual protein, and then the tube was left at room temperature for 30 min. The tube was centrifuged at 1500*g* for 10 min, and 1 mL of the clear supernatant was pipetted into a 1.7-mL microcentrifuge tube and brought to completely dryness using a heat block at 80 °C for 60 min. For the HPAEC-PAD system, the residue was re-dissolved in 1 mL of 90 mM NaOH, quantitatively transferred to a 100-mL volumetric flask, and brought to volume with 90 mM NaOH. Ten millilitres of the diluted solution was filtered through a 0.2-μm membrane (HT Tuffryn Nylon) followed by a cartridge (OnGuard II RP, Dionex, Sunnyvale, CA) to remove residual lipids, surfactants, hydrocarbons, and high molecular-weight carboxylic acids (Kadnar, 1998; Wicks, Moran, Pittman, & Hodson, 1991) prior to injection. For the HPSEC-RI system, the residue was re-dissolved in 1 mL of 0.1 M $NaNO_3$ containing 0.2% NaN_3, quantitatively transferred to a 10-mL volumetric flask, and brought to volume with 0.1 M $NaNO_3$ containing 0.2% NaN_3. The diluted solution was further purified as previously described prior to injection.

2.3. Separation and quantification of soluble sugars by HPAEC-PAD and HPSEC-RI

The HPAEC-PAD system (Dionex DX500) consisted of a GP-50 gradient pump, ED40 electrochemical detector, a CarboPac PA-10 pellicular anion-exchange resin column (250 × 4 mm i.d.) preceded by a CarboPac PA-10 guard column (50 × 4 mm i.d.) and an AminoTrap column (30 × 3 mm i.d.) (Dionex, Sunnyvale, CA). Samples were injected via an AS40 automated sampler with a 25-μL sample loop, and sugars were eluted with 90 mM NaOH at a flow rate of 1 mL/min. The mobile phase, 90 mM NaOH, was prepared by diluting carbonate-free 50% (w/w) NaOH solution in distilled water, which was previously filtered with a 0.45-μm membrane and degassed with a sonicator (Zenith Inc, T800-2H, Norwood, NJ) for 30 min. The HPSEC-RI system consisted of a 515 HPLC pump with

a 100-μL sample loop, an in-line degasser, a 2410 refractive index detector maintained at 40 °C (Waters, Milford, MA). Sugars were separated by two Shodex OHpak SB 802 HQ (300 × 8 mm) maintained at 55 °C by a column heater, preceded by a Shodex OHpak SB-G (50 × 6 mm) guard column. The mobile phase was 0.1 M $NaNO_3$ with 0.2% NaN_3 at a rate of 0.4 mL/min.

2.4. Standard curve for soluble sugar quantification with HPLC

For both HPLC systems employed, identification and quantification of the major sugars present in the samples were achieved by comparing each peak retention time and area with those of the standard. The quantity of each sugar was corrected based on the recovery ratio of the internal standard. A stock solution composed of glucose, fructose, melibiose, sucrose, raffinose, and stachyose at concentrations of 5, 5, 30, 80, 30, 80 μg/mL, respectively, was prepared for the HPAEC-PAD system. A standard curve for each sugar was prepared by injecting different concentrations of the stock solution. A second stock solution composed of glucose, fructose, sucrose, raffinose, stachyose, and maltoheptaose at concentrations of 35, 35, 1000, 600, 1000, and 800 μg/mL, respectively, were prepared for the HPSEC-RI system. Both stock solutions were passed through a 0.2-μm membrane (HT Tuffryn Nylon) and stored at −20 °C until analysis. The correlation coefficients (r) for both detectors were also checked.

2.5. Quantification of soluble sugars by enzymes

The concentrations of glucose, sucrose, and the sum of raffinose and stachyose were also determined following the raffinose-series oligosaccharides assay procedure (Megazyme RSO 8/98) with minor modifications. The extraction of the soybean soluble sugars followed the same procedure as described before. After the extraction, the whole content of the centrifuge tube was quantitatively transferred to a 50-mL volumetric flask, brought to volume with 50 mM sodium acetate buffer (pH 4.5), and then thoroughly mixed. After standing at room temperature for 15 min, 10 mL of the diluted extract was centrifuged at 1500g for 10 min. An aliquot of 0.2 mL solution was pipetted into a test tube, and glucose oxidase and peroxidase were added as described in the procedure. The absorbance of the solution was measured at 510 nm with a spectrophotometer (Bechman DU-520, Fullerton, CA), and the free glucose content was obtained using the equation in the procedure. A second aliquot of 0.2 mL solution was pipetted into another test tube and invertase was added, which hydrolyzed sucrose into glucose and fructose. Thereafter, glucose oxidase and peroxidase were added and the glucose content in the solution was determined. In a third aliquot of 0.2 mL solution, invertase and α-galactosidase were added, which converted sucrose, raffinose, and stachyose into glucose, fructose, and galactose, and the total amount of free glucose in the third solution was also measured. The amount of sucrose was calculated by subtracting the glucose in the first test tube from that of the second test tube. The amount of raffinose and stachyose was derived by subtracting the glucose in the second tube from that of the third test tube. Glucose oxidase and peroxidase were also added into the glucose standard solution (1 mg/mL) provided for the calculation of the conversion factor to convert absorbance into glucose concentration. A solution composed of glucose, sucrose, raffinose, and stachyose at concentrations of 100, 500, 500, and 200 μg/mL, respectively, was prepared and concurrently used as a control and analyzed together with the soybean extracts to check enzyme stability and method reproducibility.

2.6. Statistical analyses

Sample extraction was carried out for at least in duplicate and two measurements were done for each extracted sample for sugar quantification. Tukey–Kramer HSD test was used to detect significant difference among soybean sugars. A GLM procedure of ANOVA in version 9.0.1 of SAS statistical software (SAS Institute Inc.) was applied to detect significant differences between the extractions and the three methods (HPACE-PAD, HPSEC-RI, and enzymes) used to quantify soybean sugars.

3. Results and discussion

3.1. HPAEC-PAD

The correlation coefficients (r) of detector response vs. standard concentrations were always greater than 0.99 for all sugar standards used. A complete separation of major sugars present in the soybean extracts including the internal standard was achieved in 20 min under the condition used in the present study (Fig. 1a). Because of the sensitivity of PAD, soybean meal was diluted 1250 times for quantification. Glucose was eluted first, followed by fructose, melibiose, sucrose, raffinose, and stachyose, respectively. The amount of each sugar in the five soybean lines ranged 0.07–0.15% for glucose, 0.08–0.19% for fructose, 5.64–9.39% for sucrose, 0.25–1.35% for raffinose, and 0.29–6.33% of stachyose (Table 1). SS-516 had the largest amount of total sugars but the lowest raffinose, whereas 03CB-14 had the lowest amounts of total sugars and stachyose but the highest sucrose. Camp had the second highest amount of total sugars and the highest stachyose. Recoveries for all samples were checked and showed an average of 90.63%.

Locher and Bucheli (1998) employed a similar system with gradient elution to quantify soybean sugars in contrast to the isocratic elution in the present study. The quantification of each sugar was achieved for both studies, but isocratic elution generally encounters fewer problems of baseline re-equilibration than gradient elution.

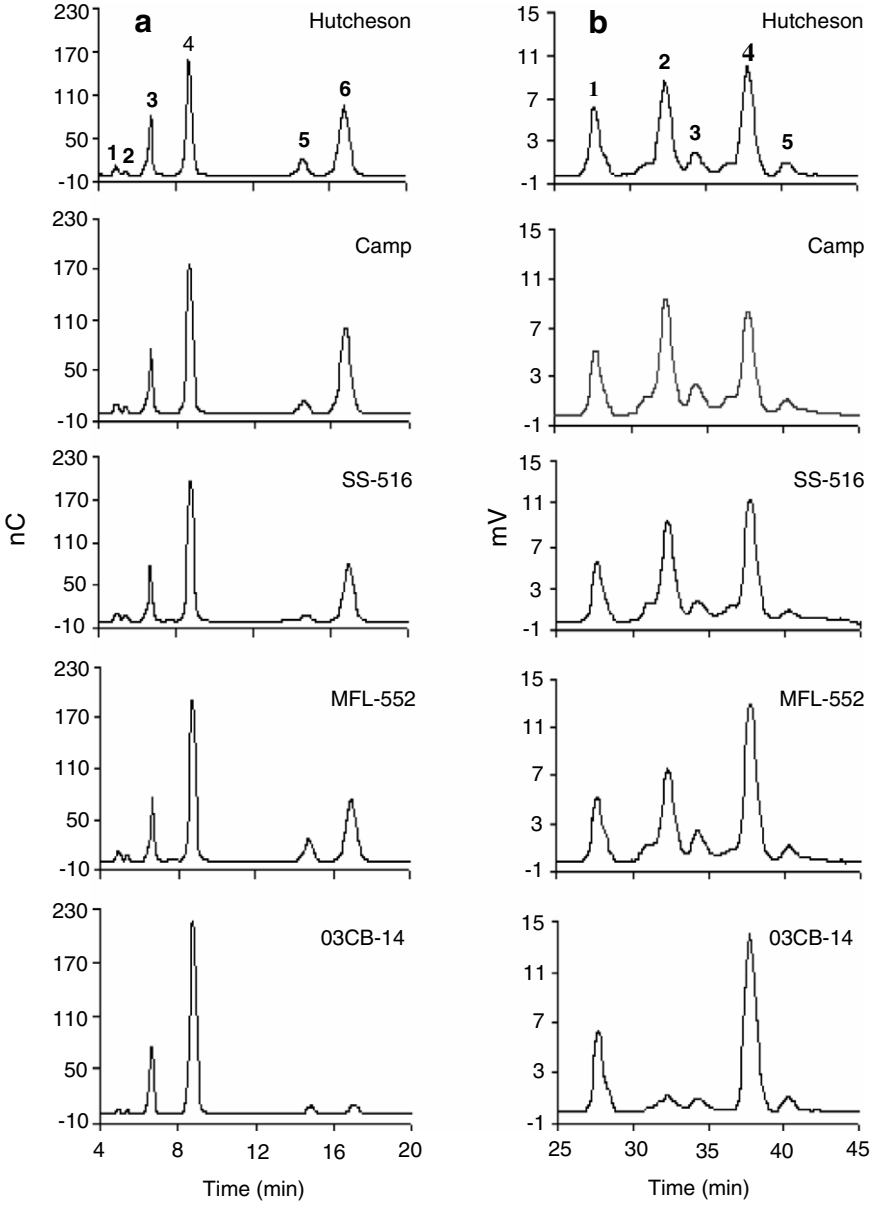

Fig. 1. Separation of sugars in five soybean lines using two HPLC systems. (a) Glucose (1), fructose (2), melibiose (3), sucrose (4), raffinose (5), and stachyose (6) with HPAEC-PAD. (b) maltoheptaose (1), stachyose (2), raffinose (3), sucrose (4), and glucose + fructose (5) with HPSEC-RI.

Table 1
Concentrations (%, db) of glucose, fructose, sucrose, raffinose, and stachyose in five soybean lines determined with HPAEC-PAD

Lines	Glucose	Fructose	Sucrose	Raffinose	Stachyose	Total sugars
Hutcheson	$0.15 \pm 0.00^{a*}$	0.08 ± 0.01^{d}	5.64 ± 0.06^{c}	1.09 ± 0.01^{b}	5.97 ± 0.06^{b}	12.93 ± 0.11^{d}
Camp	0.12 ± 0.00^{c}	0.12 ± 0.00^{b}	6.24 ± 0.06^{d}	0.84 ± 0.01^{c}	6.33 ± 0.02^{a}	13.65 ± 0.09^{b}
SS-516	0.14 ± 0.00^{b}	0.19 ± 0.00^{a}	7.91 ± 0.01^{b}	0.25 ± 0.01^{e}	5.37 ± 0.03^{c}	13.86 ± 0.03^{a}
MFL-552	0.15 ± 0.00^{a}	0.11 ± 0.01^{c}	6.97 ± 0.07^{c}	1.35 ± 0.02^{a}	4.81 ± 0.03^{d}	13.38 ± 0.07^{c}
03CB-14	0.07 ± 0.01^{d}	0.09 ± 0.00^{d}	9.39 ± 0.04^{a}	0.30 ± 0.01^{d}	0.29 ± 0.01^{e}	10.15 ± 0.03^{e}

* Mean values of at least 4 measurements in the same column followed by the same letter are not significantly different ($p < 0.05$).

3.2. HPSEC-RI

In order to improve separation, two analytical columns and a slower flow rate were used in the HPSEC-RI system based on preliminary results. Although PAD is sensitive and specific for sugar analysis, RI detection and size exclusion chromatography are universal and widely used in many analytic laboratories. Therefore, it was attempted to compare these two systems for their efficiency and reproducibility in quantifying soybean sugars. The first peak

(maltoheptaose) was eluted after 26 min and a total elution time of 43 min was needed to complete the elution of all sugars in the extracts (Fig. 1b). Because glucose and fructose have a similar molecular size, they were eluted at the same time and could not be resolved by this HPSEC-RI system. Therefore, quantification of the total amount of glucose and fructose was achieved using a standard curve comprising both glucose and fructose standards. The correlation coefficients (r) of detector response vs. standard concentrations were always greater than 0.99 for all sugar standards used, except for the standard curve prepared using the mixture of glucose and fructose with a r-value of 0.949. Because of a lower detection limit of the RI detector, samples were diluted only up to 125 times and the resulting peaks were not as well defined as those obtained with the HPAEC-PAD system.

The range of sugars quantified was 0.22–0.35% for glucose and fructose together, 5.71–9.46% for sucrose, 0.37–1.47% for raffinose, and 0.35–6.46% for stachyose (Table 2). The samples followed the same trend with respect to the amounts of total sugars and individual sugars quantified as the HPAEC-PAD system. The recoveries for all samples with this system showed an average of 91.76%, which was slightly higher than those with the HPAEC-PAD.

3.3. Enzymatic procedure

The range of sugars quantified using the specific commercial enzymatic assay procedure was 0.05–0.13% for glucose, 5.56–9.31% for sucrose, and 0.50–7.09% for raffinose + stachyose (Table 3). The results followed the same trend as the previous two HPLC systems. However, this enzymatic procedure cannot quantify fructose nor can it distinguish raffinose from stachyose. The control solution analyzed together with the samples showed 98.77% of recovery, which indicated the accuracy of the method and was used to correct for the final values. Previously, Maughan et al. (2000) reported sucrose content of 4–8.15 % in soybean using invertase and hexokinase.

3.4. Comparison of two HPLC systems and enzymatic assay

The three methods used in this study were compared for each individual sugar and the results are summarized in Table 4. The two HPLC systems quantified statistically an equal amount of sucrose for Camp, MFL-552, and 03CB-14 ($p > 0.05$). The same was observed for stachyose in 03CB-14 ($p > 0.05$). The majority of the other sugars were significantly different when quantified by the three different methods ($p < 0.001$). In general, the samples analyzed with the HPSEC-RI system showed larger sugar contents than with the HPAEC-PAD system, probably because of slightly lower resolution obtained with the HPSEC-RI. The less specific and sensitive of RI detection compared with PAD could be also responsible for the significantly higher concentrations detected.

The experiment in this study confirmed the high specificity and capability of separating and quantifying individual sugars in soybean by HPAEC-PAD (Cataldi et al., 1999; Cataldi et al., 2000; Frias et al., 1999; Mohamed & Rayas-Duarte, 1995). Refractive index detector is widely used for soybean sugar analysis when coupled with different types of columns (Black & Bagley, 1978; Black & Glover, 1980; Johansen et al., 1996; Kennedy, Mwandemele, & McWhirter, 1985; Knudsen, 1986; Kuo, VanMiddlesworth, & Wolf, 1988; Shukla, 1987). Frias et al. (1994) compared RI and PAD detectors for analyzing sugar in lentils, however, comparisons were made only regarding the detectors efficiency of analyzing small sample sizes. The use of anion-exchange column significantly decreased the elution time required to separate all the sugars and improved the peak

Table 2
Concentration (%, db) of glucose + fructose, sucrose, raffinose, and stachyose in five soybean lines determined with HPSEC-RI

Lines	Glucose + fructose	Sucrose	Raffinose	Stachyose	Total sugars
Hutcheson	$0.25 \pm 0.01^{d*}$	5.71 ± 0.02^{e}	1.15 ± 0.02^{b}	6.11 ± 0.03^{b}	13.22 ± 0.04^{d}
Camp	0.28 ± 0.01^{c}	6.29 ± 0.01^{d}	0.91 ± 0.02^{c}	6.46 ± 0.06^{a}	13.94 ± 0.05^{b}
SS-516	0.35 ± 0.01^{a}	8.01 ± 0.04^{b}	0.33 ± 0.01^{e}	5.48 ± 0.04^{c}	14.17 ± 0.07^{a}
MFL-552	0.32 ± 0.01^{b}	7.04 ± 0.07^{c}	1.47 ± 0.01^{a}	4.92 ± 0.02^{d}	13.75 ± 0.07^{c}
03CB-14	0.22 ± 0.01^{e}	9.46 ± 0.08^{a}	0.37 ± 0.02^{d}	0.35 ± 0.01^{e}	10.40 ± 0.01^{e}

* Mean values of at least 4 measurements in the same column followed by the same letter are not significantly different ($p < 0.05$).

Table 3
Concentration (%, db) of glucose, sucrose, and raffinose + stachyose in five soybean lines determined by the enzymatic procedure

Lines	Glucose	Sucrose	Raffinose + stachyose	Total sugars
Hutcheson	$0.13 \pm 0.01^{a*}$	5.56 ± 0.05^{e}	6.94 ± 0.07^{b}	12.63 ± 0.03^{d}
Camp	0.09 ± 0.01^{c}	6.16 ± 0.05^{d}	7.09 ± 0.12^{a}	13.34 ± 0.09^{b}
SS-516	0.11 ± 0.01^{b}	7.83 ± 0.04^{b}	5.48 ± 0.07^{d}	13.43 ± 0.06^{a}
MFL-552	0.12 ± 0.01^{ab}	6.89 ± 0.02^{c}	6.08 ± 0.04^{c}	13.10 ± 0.03^{c}
03CB-14	0.05 ± 0.01^{d}	9.31 ± 0.03^{a}	0.50 ± 0.08^{e}	9.85 ± 0.07^{e}

* Mean values of at least 4 measurements the same solution in the same column followed by the same letter are not significantly different ($p < 0.05$).

Table 4
Comparison of sugar concentration means in soybean seeds obtained from HPAEC-PAD, HPSEC-RI, and enzymatic analysis

System comparison	Sugars	Soybean lines				
		Hutcheson	Camp	SS-516	MFL-552	03CB-14
HPAEC-PAD vs. HPSEC-RI	Glucose + fructose	*	**	*	**	**
	Sucrose	*	—	**	—	—
	Raffinose	**	**	**	**	**
	Stachyose	**	**	**	**	—
	Total sugars	**	**	**	**	**
HPAEC-PAD vs. enzymes	Glucose	**	*	**	**	**
	Sucrose	*	*	*	*	*
	Raffinose + stachyose	**	*	*	**	**
HPSEC-RI vs. enzymes	Sucrose	*	*	*	*	*
	Raffinose + stachyose	**	**	**	**	**

* $p < 0.05$.
** $p < 0.001$.
— $p > 0.05$.

resolution. Nevertheless, the HPSEC-RI system could provide comparable results as did the HPAEC-PAD system, except for the separation of glucose and fructose.

Although statistically different, enzymes also gave comparable results to both HPLC systems, particularly the HPAEC-PAD, where similar amounts of sucrose and oligosaccharides between these two systems were obtained. With enzymes, sample preparation and quantification in the assay procedure were simple and require no expensive instrumentation. Thus, enzymatic analysis was a good alternative for analyzing small numbers of samples. On the other hand, although HPLC analysis required more steps for sample purification, separation, and system calibration, automatic sample injection allowed the continuous analysis of a large number of samples.

4. Conclusions

Statistically significant differences were observed in most soybean sugars quantified among the three systems, HPAEC-PAD, HPSEC-RI, and enzymes. Nevertheless, good estimates of the sugar contents in soybean can be achieved with all three systems. The HPSEC-RI system was shown to exhibit slightly lower capacity of separating and quantifying sugars in comparison with the HPAEC-PAD system. Enzymes presented the disadvantage of not quantifying individual raffinose and stachyose. HPAEC-PAD would be the preferred method for a complete quantification of all five major sugars in soybean. For quantification of only sucrose and the sum of raffinose oligosaccharides in soybean, the enzymatic procedure was a simple and satisfactory alternative. HPAEC-PAD is recommended for soybean breeding programs for its specificity and efficiency of separating individual sugars.

References

AACC (2000). *Approved methods of the American Association of Cereal Chemists* (10th ed.). American Association of Cereal Chemists, Methods 08-16, pp. 44–31.

Aman, P. (1979). Carbohydrates in raw and germinated seeds from mung bean and chick pea. *Journal of the Science of Food and Agriculture, 30*, 869–875.

Black, L. T., & Bagley, E. B. (1978). Determination of oligosaccharides in soybean by high pressure liquid chromatography using a internal standard. *Journal of the American Oil Chemists' Society, 55*, 228–232.

Black, L. T., & Glover, J. D. (1980). A simple and rapid HPLC analysis of sugars in soybeans and factors affecting their standardization. *Journal of the American Oil Chemists' Society, 57*, 143–144.

Cataldi, T. R. I., Campa, C., Angelotti, M., & Bufo, S. A. (1999). Isocratic separations of closely-related mono- and disaccharides by high-performance anion-exchange chromatography with pulsed amperometric detection using dilute alkaline spiked with barium acetate. *Journal of Chromatography A, 855*, 539–550.

Cataldi, T. R. I., Margiotta, G., Lasi, L., & Di Chio, B. (2000). Determination of sugar compounds in olive plant extracts by anion-exchange chromatography with pulsed amperometric detection. *Analytical Chemistry, 72*, 3902–3907.

Delente, J., & Ladenburg, K. (1972). Quantitative determination of the oligosaccharides in defatted soybean meal by gas–liquid chromatography. *Journal of Food Science, 37*, 372–374.

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

Folkes, D. J. (1985). Gas–liquid chromatography. In G. G. Birch (Ed.), *Analysis of food carbohydrate* (pp. 91–123). London, UK: Elsevier Applied Science Publisher.

Frias, J., Bakhsh, A., Jones, D., Arthur, A., Vidal-Valverde, C., Rhodes, M., et al. (1999). Genetic analysis of the raffinose oligosaccharide pathway in lentil seeds. *Journal of Experimental Botany, 50*, 469–476.

Frias, J., Hedley, C. L., & Price, K. R. (1994). Improved methods of oligosaccharide analysis for genetic studies of legume seeds. *Journal of Liquid Chromatography, 17*, 2469–2483.

Giannoccaro, E., Wang, Y.-J., & Chen, P. (2006). Effects of solvent, temperature, time, solvent-to-sample ratio, sample size, and defatting on the extraction of soluble sugars in soybean. *Journal of Food Science, 71*, C059–C064.

Hymowitz, T., & Collins, F. I. (1974). Variability of sugar content in seed of *Glycine max* (L.) Merrill and G. *soja* Sieb. and *Zucc. Agronomy Journal, 66*, 239–240.

Johansen, H. N., Glitso, V., & Knudsen, K. E. B. (1996). Influence of extraction solvent and temperature on the quantitative determination of oligosaccharides from plant materials by high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry, 44*, 1470–1474.

Kadnar, R. (1998). Determination of alkali and alkaline earth metals in oilfield water by ion chromatography. *Journal of Chromatography A, 804*, 217–221.

Kawamura, S. (1967). *Isolation and determination of sugars from the cotyledon, hull, and hypocotyls of soybeans of selected varieties. Technical Bulletin* (Vol. 15). Japan: Kagawa University.

Kennedy, I. R., Mwandemele, O. D., & McWhirter, K. S. (1985). Estimation of sucrose, raffinose and stachyose in soybean seeds. *Food Chemistry, 17*, 85–93.

Kim, S., Kim, W., & Hwang, I. K. (2003). Optimization of the extraction and purification of oligosaccharides from defatted soybean meal. *International Journal of Food Science and Technology, 38*, 337–342.

Knudsen, I. M. (1986). High-performance liquid chromatographic determination of oligosaccharides in leguminous seeds. *Journal of the Science of Food and Agriculture, 37*, 560–566.

Kuo, T. M., VanMiddlesworth, J. F., & Wolf, W. J. (1988). Content of raffinose oligosaccharides and sucrose in various plant seeds. *Journal of Agricultural and Food Chemistry, 36*, 32–36.

Ladish, M. R., & Tsao, G. T. (1978). Theory and practice of rapid liquid chromatography at moderate pressure using water as eluent. *Journal of Chromatography, 166*, 85–100.

Lineback, D. R., & Ke, C. H. (1975). Starches and low-molecular-weight carbohydrates from chick pea and horse bean flours. *Cereal Chemistry, 52*, 334–346.

Li, B. W., Schuhmann, J. S., & Wolf, W. R. (1985). Chromatography determination of sugars and starch in a diet composite reference material. *Journal of Agricultural and Food Chemistry, 33*, 531–536.

Lis, H., & Sharon, N. (1978). Soybean agglutinin-a plant glycoproteins: Structure of the carbohydrate unit. *Journal of Biological Chemistry, 253*, 3468–3476.

Locher, R., & Bucheli, P. (1998). Comparison of soluble sugar degradation in soybean seed under simulated tropical storage conditions. *Crop Science, 38*, 1229–1235.

Martens, D. A., & Frankenberger, W. T. Jr., (1990). Quantification of soil saccharides by spectrophotometric methods. *Soil Biology and Biochemistry, 22*, 1173–1175.

Maughan, P. J., Saghai Maroof, A. M., & Buss, G. R. (2000). Identification of quantitative trait loci controlling sucrose content in soybean (*Glycine max*). *Molecular Breeding, 6*, 105–111.

Mohamed, A. A., & Rayas-Duarte, P. (1995). Composition of Lupinus albums. *Cereal Chemistry, 72*, 645–647.

Molnar-Perl, I., Pinter-Szakacs, M., Kovago, A., & Petroczy, J. (1984). Gas–liquid chromatography determination of the raffinose family oligosaccharides and their metabolites present in soy beans. *Journal of Chromatography, 295*, 433–443.

Pazur, J. H., Shadaksharaswamy, M., & Meidell, G. E. (1962). The metabolism of oligosaccharides in germination soybeans, *Glycine max*. *Biochemical and Biophysical Research Communications, 99*, 78–85.

Rabel, F. M., Caputo, A. G., & Edward, T. (1976). Separation of carbohydrates on a new polar bonded phase material. *Journal of Chromatography, 126*, 731–740.

Reyes, F. G. R., Wrolstad, R. E., & Cornwell, C. J. (1982). Comparison of enzymatic, gas–liquid chromatographic, and high performance liquid chromatographic methods for determining sugars and organic acids in strawberries at three stages of maturity. *Journal of the Association of Official Analytical Chemists, 65*, 126–131.

Rocklin, R. D., & Pohl, A. (1983). Determination of carbohydrates by anion exchange chromatography with pulsed amperometric detection. *Journal of Liquid Chromatography, 6*, 1577–1590.

Shallenberger, R. S., & Moores, R. G. (1957). Quantitative determination of reducing sugars and sucrose separated by paper chromatography. *Analytical Chemistry, 29*, 27–29.

Shukla, V. K. S. (1987). Quantitative determination of oligosaccharides in defatted soybean products by high-speed liquid chromatography. *Fett Wissenschaft Technologie, 89*, 75–79.

Taira, H. (1990). Quality of soybean for processed foods in Japan. *Japanese Agricultural Research Quarterly, 24*, 224–230.

Tanaka, M., Thananunkul, D., Lee, T.-C., & Chichester, C. O. (1975). A simplified method for the quantitative determination of sucrose, raffinose and stachyose in legume seeds. *Journal of Food Science, 40*, 1087–1088.

Townsend, R. R., Hardy, M. R., Hindsgaul, O., & Lee, Y. C. (1988). High-performance anion-exchange chromatography of oligosaccharides using pellicular resins and pulsed amperometric detection. *Analytical Biochemistry, 174*, 459–470.

Trugo, L. C., Farah, A., & Cabral, L. (1995). Oligosaccharide distribution in Brazilian soya bean cultivars. *Food Chemistry, 52*, 385–387.

Wicks, R. J., Moran, M. A., Pittman, L. J., & Hodson, R. E. (1991). Carbohydrate signatures of aquatic macrophytes and their dissolved degradation products as determined by a sensitive high-performance ion chromatography method. *Applied Environmental Microbiology*, 3135–3143.