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Short communication

Development of a method to quantify sucrose in soybean grains

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ABSTRACT

The sucrose content of soybean seeds affects the final flavor of soy-derived products. The aim of this work was to develop a simple, low-cost, spectrophotometric method for sucrose quantification in soybean seeds. To achieve this goal, we combined the action of invertase, an enzyme that hydrolyses sucrose into fructose and glucose, with glucose oxidase, an enzyme widely used for glucose quantification. This system was adapted to ELISA plates, making large-scale analyses possible at low cost, with potential application in routine analyses. To validate this method, sucrose content was determined in seeds of 14 soybean cultivars by this new method, as well as by HPLC and the enzymatic method of Stitt. The correlation coefficients were high and significant between the results obtained with the new method and the HPLC method ($r = 0.9766$) and the Stiff method (0.9461).

1. Introduction

The sucrose content of soybean seeds is an important trait to improve the flavor and aroma of soy-based products, and is a critical factor during their preparation (Taira, 1990).

However, this characteristic has received little attention in the historical process of soybean breeding, which has been primarily concerned with increasing oil content, used in human consumption, and enhancing quantity and quality of the protein that is mostly used in animal feed (Cicek, 2001).

A further factor that has made breeding difficult for sucrose content in soybean seeds is the cost involved for quantifying this disaccharide (Maughan, Maroof, & Buss, 2000). There are few methodologies available for this purpose in the literature. High performance liquid chromatography (HPLC) has been used in quantitative and qualitative analysis of sucrose (Kuo, Vanmiddlesworth, & Wolf, 1988; Lowell & Kuo, 1989). In spite of the high reliability of this type of analysis, its costs are prohibitive for use in the breeding process that requires the analysis of a very large number of samples.

An enzymatic method that consists of the sequential determination of glucose, fructose and sucrose present in the soluble fraction of an alcohol extract was proposed by Stitt, Lilley, Gerherdt, and Heldt (1989). This method is less expensive than the HPLC procedure, however, the long time required to run the analyses by the Stitt method makes it unattractive. Commercial kits are also available for sucrose quantification (Kumar et al., 2010), however, it is questionable their feasibility to be used in breeding programs.

In this work we developed a method to quantify sucrose in soybean seeds with potential use in breeding programs, which enables large-scale, low-cost analyses to be carried out. This new method was adapted for use on 96-well polystyrene plates ("ELISA plates"), and is based on the combined action of invertase, an enzyme that hydrolyses sucrose into fructose and glucose, with glucose oxidase, an enzyme widely used in commercial kits to quantify glucose. To validate this new methodology, it was tested to determine the sucrose content in seed samples of 14 soybean cultivars in parallel with the HPLC and the enzymatic method developed by Stitt et al. (1989).

2. Materials and methods

2.1. Genetic materials and reagents

The samples analysed were seeds from 14 soybean genotypes obtained from the breeding program for soybean quality of the Federal University of Viçosa, Minas Gerais, Brazil. The Bioclin kit for glucose quantification based on the action of the glucose oxidase enzyme (GOD) was purchased from Química Básica Ltda, Belo Horizonte, MG, Brazil. The invertase enzyme, adenosine triphosphate (ATP) and imidazole were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The glucose-6-phosphate dehydrogenase (G6PDH), phosphoglucoisomerase (PGI) and

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hexokinase enzymes were purchased from Roche (São Paulo, SP, Brazil) and β-nicotinamide adenine dinucleotide (NAD) was purchased from Merck (Darmstadt, Germany). All the other reagents used were of analytical grade. The water used in the HPLC analyses was purified by the MilliQ System, Millipore (Billerica, MA, USA) and the analysis grade acetonitrile was filtered before use.

2.2. Carbohydrates extraction

Twenty soybean seeds from each sample were ground and then dried in a chamber for 5 h at 105 °C. The samples were then transferred to a desiccator. Using 2.0 mL microfuge tubes, approximately 20 mg of sample was weighed and 1.0 mL 80% ethanol was added to each tube, homogenised for 1 min in a vortex and placed in a water bath at 70 °C for 90 min. After this period, the tubes were centrifuged for 10 min at 16,100*g*. The supernatant was transferred to a fresh tube and the volume was completed to 1.0 mL with 80% ethanol. This extract was used for the sucrose determination by the Stitt method and by the GOD/invertase method, developed in this study.

2.3. Sucrose quantification by the GOD/invertase method

The GOD/invertase method consisted of the following procedure: in a 96-well ELISA plate, 85 μL distilled water, 5 μL alcohol extract from each sample and 10 μL invertase were placed in each well. The invertase was prepared at a concentration of 10 mg/mL in distilled water. The plate was then sealed and placed in a water bath at 55 °C for 10 min. After this period the plate was removed from the water bath and 200 μL of GOD (Bioclin kit) reagent was added, and the plate was sealed again and placed in a water bath at 37 °C for 15 min. After this time, the plate was removed from the water bath and placed at room temperature for 5 min. The absorbance at 490 nm was read in a Titertek Multiskan Plus spectrophotometer, equipped for reading ELISA plates. Standard sucrose solutions were also added to each plate in separate wells at concentrations 0%, 0.05%, 0.1%, 0.15%, 0.2% and 0.25% (g/100 mL). This allowed a calibration curve to be constructed which was used to determine the sucrose concentration in each sample. Each analysis was done in triplicate.

2.4. Sucrose quantification by the enzymatic method of Stitt

The sucrose content of the soybean seeds was also determined by the enzymatic method published by Stitt et al. (1989). The following were placed in each well of an ELISA plate: 130 μL buffer containing 200 mM imidazole, 10 mM $MgCl_2$, 4 mM NAD, 2 mM ATP and 0.4 U G6PDH; 20 μL extract (samples) and 110 μL distilled water. Glucose was measured by adding 5 μL hexokinase (0.2 U) and the reaction curve was allowed to reach a plateau. To measure fructose, 5 μL phosphoglucoisomerase (0.6 U) was added and again the reaction curve was allowed to reach a plateau. Five microlitre invertase (50 U) was added to measure sucrose. The reaction curve was allowed to reach a plateau. After reaching this last plateau, the maximum absorbance values in each plateau were recorded and the ΔA was calculated (final value minus the initial value, before adding each enzyme). Dividing this ΔA by 6.2 (NAD molar absorptivity), the absorbance value was transformed into μmol of NADH (or in glucose equivalent μmol) per well. For sucrose, the ΔA was divided by 2, and then divided by the NAD molar absorptivity. This analysis was carried out in triplicate. The readings were made at 340 nm.

The following equation was used to calculate the sucrose content:Sucrose $(\text{mg g}^{-1}\text{ dw}) = \{[(0.5 \times \Delta A/6.2) \times (\text{total extract volume/aliquot in the reaction})]/\text{seed dry weight}\} \times 0.3423$.

2.5. Sucrose quantification by HPLC

In the HPLC analysis, 20 seeds from each of the 14 soybean samples were ground in an analytical grinder, and frozen dried for 10 h in an lyophilizer. Approximately 20 mg soybean was weighed in triplicate in 2.0 mL propylene microcentrifuge tubes with screw lids. The lipids were then extracted by adding 1.0 mL petroleum ether, and heating in a water bath at 42 °C for 5 min under constant agitation. After this period the samples were homogenised in a vortex and centrifuged at 16,100*g* for 10 min and the petroleum ether phase was discarded. This procedure was repeated five times. After extracting the lipids, the soluble sugars were extracted by adding 1.0 mL 80% ethanol to each tube, that were heated in a boiling water bath for 5 min, under agitation. After this period the samples were allowed to cool to room temperature, and were then homogenised and centrifuged at 16,100*g* for 5 min. The alcohol solution was collected in a 10 mL beaker. This procedure was repeated three times. After extracting the sugars, the beaker was placed in a chamber at 48 °C until all the solvent had evaporated. Then the sugars were suspended in 1 mL 80% ethanol, and the solution was transferred to an Eppendorf tube and kept at −20 °C. Before application, the samples were thawed, centrifuged at 16,100*g* for 10 min and filtered. Aliquots of 25 μL were analysed in a Shimadzu chromatograph with a refraction index detector. The mobile phase used was acetonitrile:water (80:20). A Supelcosil LC-NH_2 Supelco column, was used. Aiming mainly to quantify the sucrose in the samples, standard solutions were also applied containing known quantities of the sugars fructose, sucrose, raffinose and stachyose. The sucrose concentration in each sample was determined by a calibration curve.

2.6. Correlations between the methods

Pearson correlation coefficients estimates were determined between the three methods used for sucrose quantification. The following expression was used:

$$r_{x_1x_2} = \frac{\text{cov}(x_1, x_2)}{\sqrt{\text{var}(x_1)\text{var}(x_2)}}$$

where $r_{(x_1,x_2)}$ = estimator of the correlation coefficients between the sucrose concentration determined by methods 1 and 2. Cov(x_1,x_2) = estimator of the covariance between the sucrose concentration determined by methods 1 and 2. var(x_1) e var(x_2) = estimators of the variances in the sucrose concentration determined by methods 1 and 2, respectively.

3. Results and discussion

For sucrose determination, we combined the action of invertase and glucose oxidase. This system was adapted to 96-well polystyrene plates. Sucrose determination was based on the following combined reactions:

$$\text{Sucrose} \xrightarrow{\text{Invertase}} \text{Glucose} + \text{Fructose}$$

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{Phenol} \rightarrow \text{Benzoquinone (pink colour} - A_{490\text{nm}})$$

In order to validate this new method, the sucrose content in soybean seeds was determined and compared with values obtained by HPLC and the enzymatic method of Stitt, two widely procedures used for sucrose quantification.

The sucrose concentrations determined by these three methods and their respective coefficients of variation are shown in Table 1. Sucrose concentration in the seeds varied from 2.84% to 7.28%, in

Table 1
Sucrose concentrations (mean of three replications) in % (g/100 g) in different samples of soybean grains, determined by the HPLC, GOD/invertase (GOD/INV) and Stitt methods. The coefficient of variation (CV) for each method is also shown.

	HPLC		GOD/INV		Stitt	
Sample	Sucrose (%)	CV (%)	Sucrose (%)	CV (%)	Sucrose (%)	CV (%)
CD2053PTA.74.B PL20	2.69 ± 0.40	14.88	2.84 ± 0.34	11.97	7.96 ± 0.62	7.78
CD2053PTA.61.22.4 PL8	4.70 ± 0.41	8.72	4.14 ± 0.28	6.77	9.01 ± 0.43	4.77
CD2053PTA74B.1.1	5.89 ± 0.18	3.06	6.16 ± 0.30	4.87	9.81 ± 0.71	7.24
CD2053PTA152.7.1	5.29 ± 0.22	4.20	5.31 ± 0.41	7.72	10.06 ± 0.82	8.15
CD2053PTA350.35.6.22.1	3.94 ± 0.21	5.32	3.86 ± 0.33	8.56	8.61 ± 0.78	9.06
CD2053PTA295.7.3.3.1	3.91 ± 0.52	13.41	4.61 ± 0.43	9.33	8.92 ± 0.67	7.51
CD2013PTA106A PL4	3.75 ± 0.37	9.74	3.67 ± 0.31	8.45	7.96 ± 0.96	12.06
CD2013PTA171.20.7 PL7	3.33 ± 0.38	11.51	3.86 ± 0.18	4.66	8.13 ± 0.28	3.45
WILAMI	7.90 ± 0.35	4.46	7.12 ± 0.49	6.88	10.55 ± 0.58	5.50
NATTO	5.74 ± 0.12	2.09	5.55 ± 0.67	12.08	9.62 ± 0.9	9.35
LATEGIANT	7.87 ± 0.39	4.93	6.64 ± 0.44	6.62	11.10 ± 1.15	10.36
TADACHA	8.63 ± 0.20	2.28	7.28 ± 0.54	7.42	11.57 ± 0.64	5.53
TOFU	6.98 ± 0.76	10.92	6.04 ± 0.36	5.96	9.80 ± 0.85	8.67
TAMBACURA	7.80 ± 0.54	6.90	7.27 ± 0.57	7.84	10.98 ± 0.63	5.74

Table 2
Estimates of the Pearson correlation coefficients among the different methods used in sucrose quantification in soybean seeds.

Methodology	Correlation coefficient
HPLC × GOD/INV	0.9685[b]
HPLC × GOD/INV[a]	0.9854[b]
HPLC × Stitt	0.9579[b]
Stitt × GOD/INV	0.9461[b]

[a] Analysis performed on the same alcohol extract used in the HPLC.
[b] Significant at 5% probability by the "t" test.

agreement with values cited by Kumar et al. (2010). The highest value for sucrose concentration was observed in cultivar Tadacha for the three methods tested. Our results show that there was consistency between the GOD/invertase method and those regularly used for sucrose determination. In addition, the GOD/invertase method is highly reproducible with coefficients of variation ranging from 4.87% to 12.08% (Table 1).

The correlation coefficients between the methods are shown in Table 2. The GOD/invertase method presented a high correlation coefficient with the HPLC method. The value was 0.9685 when two different extract preparations were analysed, but this value increased to 0.9858 when the extract prepared for HPLC analysis was also used in the GOD/invertase method. The correlation between the GOD/invertase method and the enzymatic method of Stitt was also high ($r = 0.9461$). The high correlation coefficient values obtained demonstrate the accuracy and robustness of the GOD/invertase method.

It is important to notice that although the new method has been developed to quantify sucrose in soybean seeds it can be used for other types of biological samples. In the case of soybean the amount of free glucose is negligible (Hou, Chen, Shi, Zhang, & Wang, 2009), however, the amount of free glucose should be considered when adapting this procedure to other types of biological materials. A control without addition of invertase would be necessary when free glucose is present.

4. Conclusions

The method developed requires basically a spectrophotometer adapted for reading ELISA plates and low-cost reagents. It is an unexpensive alternative for sucrose quantification analyses in soybean breeding programs and can be easily adapted to other species, allowing low cost large-scale analyses.

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