Determination of Oligosaccharides in Soybeans by High Pressure Liquid Chromatography Using an Internal Standard

L.T. BLACK and **E.B. BAGLEY**, Northern Regional Research Center,
Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61601

ABSTRACT

A method was developed for the quantitative analysis of oligosaccharides in soybeans by high pressure liquid chromatography (HPLC). The sugars were extracted from the soy flour using an ethanol-water solution. Separation of the oligosaccharides was effected by injecting a sample extract onto an HPLC equipped with a μBONDAPAK/carbohydrate® column. Three quantitative techniques were investigated for determining the separate sugars: (a) repetitive injection (i.e., alternately injecting equal volumes of standards and samples); (b) extract analyzed before and after spiking with raffinose (one of the unknowns); (c) the addition of a pure inexpensive internal standard, β-cyclodextrin, which was separated completely from the other oligosaccharides. These methods were successfully applied to the quantitative analyses of two varieties of soybeans and other soybean products such as soy milk and soy protein concentrate.

INTRODUCTION

Smiley et al. (1) have studied the treatment of soybean whey in milk by the hollow fiber enzyme reactor technique (HFER), using α-galactosidase to reduce the levels of raffinose and stachyose. These oligosaccharides have been considered to be responsible for gastrointestinal distress associated with the food uses of whole soybeans. This study is being continued, since elimination of the problems associated with oligosaccharides will serve to increase the potential of soybeans for human nutrition.

An accurate and reasonably fast analysis for oligosaccharides was needed in order to control the HFER. Several methods for the quantitative determination of oligosaccharides have been published in recent years. These methods are generally lengthy and tedious and often are not reproducible. Albon and Gross (2) were among the first to use paper chromatography (PC) for the quantitative analysis of oligosaccharides. The work was extended by Kawamura (3) and Tanusi et al. (4). Tanusi separated the sugars by PC, then extracted the separate sugars from the paper and subsequently determined the sugars by colorimetry. The separation by PC is lengthy and is followed by the rather tedious steps of extraction and color formation.

Hymowitz et al. (5) extracted the sugars from defatted soy flour with ethanol-water, removed the extraction solvent in a forced-draft oven, and silylated the sugars with Tri-Sil Z®. The silyl ethers were then quantitatively determined by gas chromatography (GC). Delente and Ladenburg (6) used essentially the same silylation procedure as Hymowitz, but employed a simpler, more rapid extraction technique. Many workers have employed various silylating reagents with GC with varying degrees of success. It has been the experience of several workers at this laboratory, as well as others, that either the formation of these oligosaccharide silyl ethers is not always quantitative, or thermal degradation occurs because of the necessarily high injection port temperature of the GC. It is conceivable that thermal breakdown of these high molecular weight silyl ethers might be catalyzed by residual material deposited on the injection port from various other types of samples previously injected on the GC.

Walker and Saunders (7) successfully employed cation-exchange resins in conjunction with column chromatography to separate oligosaccharides. The quantitation of these separated sugars requires the collection of dozens of fractions and is quite lengthy.

Tanaka et al. (8) successfully determined oligosaccharides quantitatively using thin layer chromatography (TLC) followed by extraction and colorimetry. Here again this method requires some very tedious operations working with extremely small quantities of sugars.

High pressure liquid chromatography (HPLC) has already seen several applications in the field of sugar separations. Brobst et al. (9) separated oligosaccharides by HPLC using a polyacrylamide gel system. Linden and Lawhead (10) accomplished saccharide separations with HPLC using it in conjuction with a Technicon Auto Analyzer. Conrad and Palmer (11) and Rabel et al. (12) considered the adaptation of HPLC to carbohydrate analysis in general, and specifically showed the separations of soy oligosaccharides.

Cegla and Bell (13) analyzed oligosaccharides on oilseed flours using a combination of TLC and HPLC. TLC was employed to remove organic matter prior to HPLC to increase column life. The lengthy and tedious TLC clean-up could hardly be justified by the increased column life. The removal of organic matter from the extract was not absolutely necessary since none of the extracted organic matter yielded any artifact peaks. Previous work reporting the separation of oligosaccharides by HPLC has not described in detail methods for actual sugar quantitation. Thus, the purpose of this paper is to describe the techniques we developed to determine quantitatively those oligosaccharides in soybeans and soy products by HPLC.

Three different methods for oligosaccharides quantitation were studied: (a) the often used techniques of repetitive injection of samples and standards, (b) spiking an aliquot of the sample extract with known quantities of raffinose, one of the unkowns, followed by HPLC analysis of both the spiked and unspiked extracts, and (c) the addition of pure, inexpensive internal standard which completely separated from the oligosaccharides and had a response factor similar to the oligosaccharides.

EXPERIMENTAL PROCEDURES

To prepare a sample for analysis, 1.00 g of ground defatted soybean meal was weighed into a 50-ml polyethylene centrifuge tube (100 x 26 mm). The sample was thoroughly mixed with 10 ml of ethanol-water (80:20, v/v) using a glass stirring rod. The sample was heated in a 80 C water bath for 30 min with frequent stirring (a vortex mixer may be used) and centrifuged at 2000 rpm for 3 min. The above extraction was repeated three more times, each time combining the extracts in a 50-ml beaker. The combined extract was deproteinized with 2 ml of 10% lead acetate and centrifuged. The precipitate was washed with 3 ml of the ethanol solution and recentrifuged. Wash and extract were com-

bined. The extract was evaporated to ca. 20 ml on a steam plate. Excess lead was precipitated with 10% oxalic acid until the extract was free of lead. The extract was then centrifuged to remove the lead oxalate, and the clear extract was quantitatively transferred into a 25-ml volumetric flask and brought to volume with water.

Chromatographic Separation

The liquid chromatograph used was Waters Associates model ALC 201 equipped with a differential refractometer. The separation was achieved on a μBONDAPAK/carbohydrate® column (30 cm x 4 mm ID packed with 10-μsilica with an amine functionality) (Waters Associates, Milford, MA). The elution solvent was acetonitrile-water (70:30, v/v) with a pump rate of 2.0 ml/min. A 10-μl sample of the extract was injected (using a 25-μl Pressure-Lok microsyringe, Precision Sampling Corporation, Baton Rouge, LA) into a model U6K injector, which allows the sample to be put on a by passed injection port (no pressure exists in the bypassed state). The by pass valve was then switched to introduce the sample to the pressurized column.

The retention times for oligosaccharides and standards were: (a) sucrose, 4.5 min; (b) raffinose, 7.5 min; (c) stachyose, 13 min; and (d) β-cyclodextrin, 15 min. The column was ready for injection of the next sample in 17 min.

Methods of Quantitation

Repetitive injection: Using this technique, repeat samples of exactly 10.0 μl of a standard water solution of sucrose, raffinose, and stachyose (all obtained from ICN Pharmaceuticals, Plainview, NY), containing ca. 20 mg/ml of each sugar accurately weighed and corrected to a dry basis, were injected into the chromatograph until reproducible peak areas were obtained. Samples of 10.0 μl of each extract were likewise injected until reproducible peak areas were obtained. Between every two or three samples of extract, a standard was injected. The average value obtained for peak area/mg of each standard sugar was used to calculate amounts of the unknown sugars:

$$\text{Sucrose} = (\text{ASu})\ (s)\ (v)\ (100)/(\text{ASu}')\ (W),$$

where ASu = peak area of unknown sucrose, s = mg sucrose/10.0 μl standard, v = final volume-(ml), ASu′ = peak area of standard sucrose, and W = sample weight-(g).

Raffinose and stachyose are calculated in the same manner by substituting the corresponding peak areas and standard concentrations. Integration of peak areas was accomplished by triangulation or computer assist.

Spiking with raffinose: Approximately 200 mg of raffinose was accurately weighed into a 10-ml volumetric flask. Sample extract (8-9 ml) was transferred into the flask containing the raffinose. Raffinose dissolved in the sample extract in about 15 min with intermittent shaking. The raffinose spiked solutions were brought to volume with sample extract. Approximately 10 μl of sample extract was injected into the HPLC. After all peaks from the sample extract were eluted (ca. 15 min), 10 μl of raffinose spiked sample extract was injected into the HPLC. Finally, 10 μl of a water solution of the standard sugars was injected into the HPLC, from which response factors (Rf) were calculated. After determining peak areas, the individual sugars were calculated using the following equation:

$$(100)\ (v)/(1000)\ (W) = I,$$

where v = final volume-(ml), W = sample weight-(g).

$$\%\ \text{Sucrose} = (I)\ [r/Rf(\tfrac{A'R}{A'Su} - \tfrac{AR}{ASu})],$$

where r = added raffinose-(mg), Rf = response factor for sucrose, A = area for the unspiked sample, A′ = area for the spiked sample, R = raffinose peak area, Su = sucrose peak area, St = stachyose peak area. To calculate % stachyose, replace ASu and A′Su with ASt and A′St and substitute the Rf value for stachyose. The raffinose may be calculated by ratio-proportion from either the % sucrose to peak area or % stachyose to peak area.

Internal standard: Approximately 200 mg of β-cyclodextrin (cycloheptaamylose, obtained from Aldrich Chemical Company, Milwaukee, WI) was accurately weighed into a 10-ml volumetric flask. β-Cyclodextrin was dissolved in 8-9 ml of sample extract and sample extract was added to volume. Approximately 10 μl of this sample was injected into the HPLC; all peaks were eluted in about 17 min. A standard water solution was prepared containing 20 mg/ml each of sucrose, raffinose, stachyose, and β-cyclodextrin accurately weighed on a dry basis (the water content of each sugar was measured by Karl Fisher titration). Approximately 10 μl of this standard was injected, and the response factors were calculated for each peak. After determining all peak areas, individual sugar percentages were calculated from the following equation:

$$\%\ \text{Sucrose} = (IS)\ (Rf)\ (ASu)\ (100)/(AIS)\ (W),$$

where IS = weight of internal standard, Rf = response factor for sucrose, ASu = peak area of unknown sucrose, AIS = peak area of internal standard, W = sample weight-mg.

To calculate % raffinose or stachyose, replace ASu with AR (peak area of unknown raffinose) or ASt (peak area of unknown stachyose) and substitute the corresponding Rf value.

RESULTS AND DISCUSSION

Three basic considerations must be dealt with when undertaking the analysis of oligosaccharides in soybeans by HPLC. These are: (a) the extraction of sugars from the soy flour, (b) the separation and detection of these sugars by HPLC, and (c) their accurate quantitation. Upon consideration of existing methods for extraction of oligosaccharides from soy flour, it was found that the extraction procedure using ethanol-water (80:20, v/v) similar to that used by Hymowitz et al. (5) was satisfactory with one minor exception. Sodium bicarbonate or carbonate when used for precipitation of excess lead was responsible for two artifact peaks that were eluted at the same time as sucrose and made quantitation of sucrose impossible. However, by changing the precipitant to oxalic acid, a reagent blank could be prepared which contained no artifact peaks. Removal of all sugars was found to be complete after four extraction. This evidenced by the lack of any oligosaccharides in subsequent extracts as determined by both HPLC and TLC. Celga and Bell (13) contend that ethanol and water solutions do not completely remove all soluble sugars since the extracted residue reacted positively to the Dubois et al. (14) phenol-sulfuric acid colorimetric assay. The fact that exhaustively extracted soy flour residue contains substances that react positively to phenol-sulfuric acid does not indicate the presence of unextracted oligosaccharides; rather it simply indicates there are more complex polysaccharides remaining, as would be expected.

The separation and detection of the oligosaccharides in soybean flakes by HPLC has been described by Conrad and Palmer (11), using a μBONDAPAK/carbohydrate® column and refractive index detector. We confirm that Conrad's procedure is satisfactory. The accuracy and reproducibility of the HPLC was determined by injecting water solutions containing known quantities of each oligosaccharide (d.b.) present in soybeans. Both the accuracy and precision of the HPLC were excellent as seen in Table I.

None of the existing methods employing HPLC for the determination of oligosaccharides in beans details a proce-

TABLE I

Standard Sugars Added Versus % Found by HPLC[a]

Standard Sugars	% (d.b.)[b]	Day-1[c]	Day-2	Day-3	Response factors[d]
Sucrose[a]	48.2	48.0	48.2	48.1	1.13
Raffinose[a]	11.4	11.9	11.4	12.0	1.00
Stachyose[a]	40.4	40.2	40.4	39.9	1.10
Sucrose	44.0	44.3	44.0	44.1	1.11
Raffinose	18.4	18.2	18.4	18.5	1.00
Stachyose	37.7	37.5	37.7	37.4	1.07
Sucrose	36.0	35.8	36.2	36.1	1.08
Raffinose	34.0	33.6	33.4	33.3	1.00
Stachyose	30.0	29.9	30.4	30.6	1.03
Sucrose	32.0	32.0	32.4	32.4	1.07
Raffinose	40.3	39.6	39.4	39.4	1.00
Stachyose	27.7	28.4	28.2	28.2	1.03

[a]Standard deviation: sucrose 0.177, raffinose 0.206, and stachyose 0.254.
[b]Dry basis.
[c]Standard sugars analyzed on three different days.
[d]Average response factors: 1.10, 1.00, 1.06.

TABLE II

Relative Area[a] from Ten Repetitive Injections[b] (10 μl) of a Standard Solution of Oligosaccharides

Sucrose	Raffinose	Stachyose
1965[c]	1753[c]	1686[c]
1969	1782	1732
1990	1785	1735
2023	1814	1769
2055	1837	1787
2031	1843	1874
2061	1878	1908
2080	1854	1799
2024	1850	1801
2005	1790	1745

[a]Overall standard deviation 50.3 (RSD = 2.7%).
[b]Run over an 8-hr period.
[c]Relative peak area values.

TABLE III

HPLC Analysis by Added Raffinose of Two Varieties of Defatted Soybeans[a]

Sugar	Day-1[b]	Day-2	Day-3
		Amsoy	
Sucrose	7.2[c]	7.0	7.0
Raffinose	1.0	1.1	0.9
Stachyose	4.9	4.9	4.5
		Beeson	
Sucrose	7.4	7.6	7.1
Raffinose	1.1	1.1	1.0
Stachyose	4.8	4.6	4.7

[a]Standard deviation—both varieties: sucrose 0.184, raffinose 0.079, and stachyose 0.165.
[b]Samples analyzed on three different days.
[c]Values reported in % d.b.

dure for quantitation. In this study we have examined three means of quantitatively measuring oligosaccharides in soybean extracts by HPLC.

Repetitive Injection

Many workers using HPLC obtain poor quantitation by comparing peak areas of repetitive injections from both samples and sugar standards. The errors associated with repetitive injection are: (a) the precise injection of small quantities of sample is very difficult, and (b) detector responses fluctuate with time. The following experiment was carried out to check these factors. Precisely 10.0 μl of a water standard solution containing 20.5 mg/ml sucrose, 17.6 mg/ml raffinose, and 18.1 mg/ml stachyose (all sugars on a dry basis) was injected onto an HPLC column ten times over an 8-hr period.

The data shown in Table II were obtained by computer assist and represent relative area only. The average relative standard deviation for the three sugars was 2.7%, while the least significant difference was 162 for a single injection. There was no statistical basis for the rejection of data from any single injection. From the data, it is obvious that the area increases with time, and, therefore, the repetitive injection technique must be continuously standardized (i.e., interposing sample injections with standard injections). Inability to inject reproducible amounts onto the HPLC column is an inherent variable in this procedure.

Internal Standard-Spiking with Raffinose

The use of an internal standard with each soy sample extract is undoubtedly a better means of quantitation than repetitive injection. The use of one of the unknowns as the internal standard is quite plausible and can be accomplished by the addition of a known quantity of one of the oligosaccharides in question. Raffinose was selected for this purpose since the concentration of raffinose in soybeans is relatively low, and high purity raffinose is available. The addition of raffinose as the internal standard to an aliquot of the sample extract obviously required that both the extract and spiked sample extract be analyzed.

The justification for the use of raffinose as the internal standard is seen in Table I. As the increments of raffinose are increased in each standard, the response factors for the other sugars remained essentially the same. Thus, the quantity of raffinose used to spike the sample extracts did not introduce any error.

When this technique is employed, it is necessary to run only one standard for determination of the Rf (such as the values listed in Table I).

The principal value of using raffinose as an internal standard instead of a higher molecular weight internal standard occurs when the HPLC column begins to lose efficiency. Even a column with a greatly reduced number of theoretical plates will still separate oligosaccharides, while higher molecular weight internal standards began to overlap the stachyose.

Using this procedure, oligosaccharide compositions were obtained for two varieties of defatted soybeans as shown in Table III.

Internal Standard-Separate Compound

A final approach to the quantitation of oligosaccharides in soybeans by HPLC was to locate an internal standard which could be completely separated from all the sugars in the soybean extract, thus eliminating repetitive injection or the need for spiking a sample with raffinose and running it twice. The use of a separate compound as an internal standard has been described by Dal Nogare and Juvet (15).

After examining several classes of compounds, it became apparent that low molecular weight compounds would be unsatisfactory due to the inability of the isocratic solvent system (acetonitrile-water) to separate them from the monomers (fructose and glucose) and dimer (sucrose). In addition, as the number of theoretical plates of a column decreased with use, separation of these low molecular weight compounds decreased. A chromatogram showing the separation of the oligosaccharides present in a soy flour extract (including the added internal standard) is shown in Figure 1.

If, however, the solvent system was changed to effect a separation of the low molecular weight carbohydrates, the time required for the elution of the higher molecular weight oligosaccharides became prohibitive. The considerations for choosing a higher molecular weight internal standard involved availability, cost, and purity. The following relatively pure and inexpensive compounds were tried as internal standards: (a) maltohexaose, (b) cyclohexaamylose, and (c) β-phenyl-D-glucoside. None of these compounds separated completely from the stachyose, which made then unsatisfactory as internal standards. It was found that baseline separation between stachyose and β-cyclodextrin (also known as cycloheptaamylose and β-Schardinger dextrin) could be achieved using the same HPLC conditions described above. The response factor of β-cyclodextrin was 1.03 similar to the other oligosaccharides (the same as stachyose). Since the β-cyclodextrin could be separated from the oligosaccharides, was relatively inexpensive, and was readily soluble in the soybean extract, it was ideally suited as an internal standard.

Another major advantage in using an internal standard which elutes after the last sugar of interest would be to prevent the internal standard from covering a peak which might be formed due to hydrolysis of either raffinose or stachyose, i.e., melibiose. The same two varieties of defatted soy flour as shown in Table III were analyzed using β-cyclodextrin as the internal standard with the results comparing very favorably for all oligosaccharides on both varieties (Table IV). The method was also applied to soy fractions containing much higher concentrations of oligosaccharides (Table V).

After several dozen injections, the number of theoretical plates of the HPLC column began to decrease until the β-cyclodextrin started overlapping the stachyose. This reduction in column efficiency is due to an irreversible loss of active sites caused by contaminants found in organic materials. The removal of these impurities can be accomplished very simply by using a guard column (a 3-cm μBONDAPAK precolumn) which obviates the need for any sample pretreatment for the removal of organic matter. As the column became less efficient, the peaks could continue to be separated by decreasing the water content of the elution solvent (decreasing solvent polarity). Over a period of time, the column pressure gradually increased due to small deposits of protein or other organic matter which came through the guard column. These deposits were easily removed (since they were water solubles from the samples) by pumping water through the column until the pressure was substantially reduced.

The chromatograms shown by Conrad and Palmer (11) for soybean flake extract show peaks which are referred to as higher saccharides. Kawamura (3) has stated that soybeans contain a trace of verbascose. Quantitative data on soybean varieties that we have analyzed during this study indicate the presence of verbascose under 0.05%.

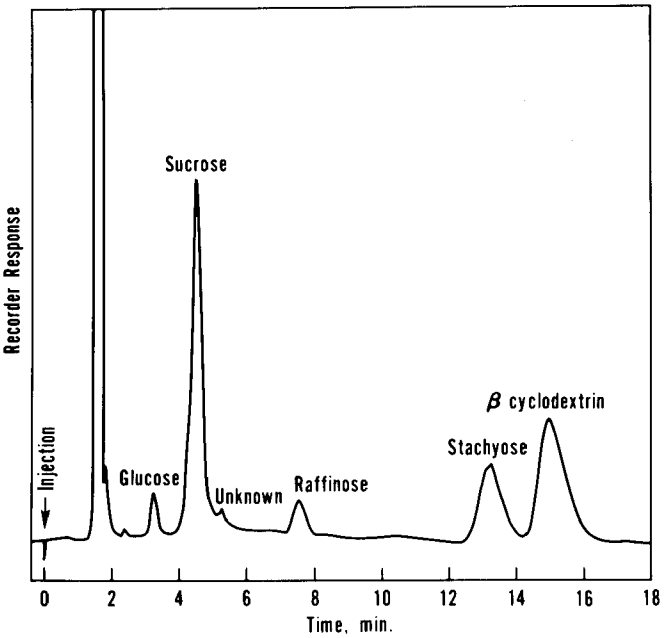

FIG. 1. Chromatogram of a defatted soy flour extract including the internal standard.

TABLE IV

HPLC Analysis of Two Varieties of Defatted Soybeans Using β-Cyclodextrin as an Internal Standard[a]

Sugar	Day-1	Day-2	Day-3
		Amsoy	
Sucrose	7.6[b]	7.3	7.6
Raffinose	1.1	0.9	1.2
Stachyose	4.9	4.8	4.8
		Beeson	
Sucrose	7.5	7.5	7.4
Raffinose	1.1	1.2	1.2
Stachyose	4.6	4.8	4.9

[a]Standard deviations—both varieties: sucrose, 0.115; raffinose, 0.105; and stachyose, 0.105.
[b]Values reported in % d.b.

TABLE V

Oligosaccharides Obtained from a Soy Whey Replicate Analysis by HPLC Using an Internal Standard[a]

Sucrose (% d.b.)	Raffinose (% d.b.)	Stachyose (% d.b.)
23.4	5.5.1	15.6
23.1	4.0	15.8
22.6	3.7	15.1
23.1	3.5	15.9
23.6	3.7	16.4

[a]Standard deviation: sucrose 0.378, raffinose 0.640, and stachyose 0.472.

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