High Pressure Liquid Chromatography for the Analysis of Soluble Carbohydrates in Defatted Oilseed Flours[1]

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ABSTRACT AND SUMMARY

The soluble carbohydrate content of four defatted oilseed flours (cottonseed, soybean, peanut, and sunflower) was determined by using high pressure liquid chromatography. The best method of separating the sugars from the other components of water extracted flour was found to be thin layer chromatography. Plates of Silica Gel H with a running solvent of isopropanol-water-ehtyl acetate (54:26:20) gave good isolation of the sugars. The μ BONDAPAK/ Carbohydrate column was used to analyze the sugars. An eluting solvent of acetonitrile and water (75:25 and 72:28) at a flow rate of 1.5 ml/min gave the best resolution of the carbohydrates. The major carbohydrates in the oilseed flours were glucose, sucrose, raffinose, and stachyose. Small amounts of trehalose and an unidentified disaccharide were found in the flour of sunflower.

INTRODUCTION

There is an increasing interest in using defatted oilseed flours in food products. Today these flours, especially those of soybean origin, are rapidly becoming an integral part of food products.

Defatted oilseed flours are composed essentially of protein and carbohydrate. Two different types of carbohydrates are present in the oilseeds, these being soluble mono- and oligosaccharides and insoluble polysaccharides. It has been found that the soluble sugars are the main cause of the flatulence problem (1). It follows that there is a need for a simple, accurate analytical method for determining the carbohydrate composition of these potential food ingredients. Such a method would also be useful in studies of fermentation processes and the reactions of carbohydrates.

There are many methods for analyzing mono- and oligosaccharides. However, each of the following methods has its limitations.

The colorimetric method (2) gives only a reliable estimate of the sugar content of a pure solution or of a known mixture of sugars. The gravimetric (3) method can be used to determine the total sugar content in a sample, but it does not distinguish between different carbohydrates. Thin layer chromatography (TLC) (4) and paper chromatography (PC) (5) are useful qualitative methods, but results are difficult to quantitate. Gas liquid chromatography (GLC) (6) is one of the most accurate methods; however, this method requires derivatization of the sugars. The enzymatic procedure (7) is highly specific, but the reagents required for large numbers of analyses are expensive.

High pressure liquid chromatography (HPLC) removes some of the limitations imposed by the other methods of analysis. It provides a means for the rapid analysis of a large spectrum of saccharides and requires a minimum of sample preparation. HPLC was found to be an adequate method for determining the carbohydrate content in hydrolysates of various cellulosic wastes and in apple cider (8), juices (9), corn syrup (10), and wine (11). However, none of these procedures deals with a situation as complex as that found with oilseed flours, where the soluble carbohydrates as natural components first have to be separated from both soluble and insoluble indigenous substances which would interfere with the carbohydrate chromatogram.

As this work was being finalized for publication, an article on the analyses of carbohydrates by HPLC (12) has appeared. Among other procedures this publication described the analyses of sugars in food legumes. The method describes extraction of the soybean with ethanol-water (80:20) followed by lyophilization of the extract. The filtered extract was injected into the HPLC instrument without further pretreatment. There may be some serious questions about this approach. First of all, it seems probable that the other components extracted with the solvent will interfere in the chromatogram. Extracted proteins, peptides, or amino acids would also rapidly decrease the efficiency of the expensive analytical column. Finally, the data of the present work indicate that ethanol-water (80:20) does not efficiently extract the sugars from oilseed flours and would result in a misleading quantitation of the sugar content.

The objective of this work was to determine the carbohydrate content of the flours of four defatted oilseeds—glandless and deglanded by the Liquid Cyclone Process (LCP) (13) cottonseed, peanut, soy, and sunflower. HPLC was found to be the best method to accomplish this objective.

EXPERIMENTAL PROCEDURES

Equipment and Column Packing

High pressure liquid chromatograph: Waters Model ALC-200, equipped with a Model 6000A solvent delivery system; R-400 differential refractometer detector sensitive to a change of 1 X 10^{-7} refractive index units, attenuation 1/4 - 64X; and a Model U6 K Universal Injector, Water Associates, Inc. Milford, MA.

Strip Chart Recorder: Hewlett Packard 7123A, 10 mV full scale with chart speed of 15 in./hr. Integrator: Hewlett Packard Model 3373B. Bio-Gel HTP (Hydroxylapatite); Bio-Gel P-2, P–4 and P-6 (all 200 -400 mesh) gel permeation resin, Bio-Rad Laboratories, Richmond, CA. A normal phase liquid chromatography packing material—Corasil II; a strong anion exchanger—Bondapak AX/Corasil; and a μ BONDAPAK/carbohydrate, stainless steel column (30 cm X 4 mm ID), Water Associates.

Materials

The sugar standards were of the highest purity available from Supelco, Inc., Bellefonte, PA. Acetonitrile of spectrophotometric grade was obtained from Burdic and Jackson Laboratories, Inc., Muskegon, MI. The water was distilled in glass, deionized, and redistilled. Silica Gel H Type 60 was obtained from E. Merck Reagents, Elmsford, NY (30 g of material in 60 ml water was used for five TLC plates, 20 X 20 cm, 0.25 mm thick).

Defatted oilseed flours from glandless cottonseed, experimental (Spanish) peanuts, and sunflower seed were produced in the pilot plant at the Oilseed Products Laboratory, Texas A&M University, College Station, TX. The rest of the flours were industrial products: LCP, Plains Cooperative Oil Mill, Lubbock, TX; commercial (Virginia) peanuts, Gold Kist, Atlanta, GA; and soybean (Soya Fluff,

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200W), Central Soya, Chicago, IL.

Procedure

The flours were extracted in a 1:15 solid to solvent ratio at 50 C with three different solvent systems. Ethanol-water (80:20 and 60:40) and water were used as solvents. The slurries were centrifuged and the supernatants decanted. This procedure was repeated with the residue two additional times. The combined supernatants were evaporated in vacuo or freeze-dried.

These extracts still contained large amounts of protein and other natural products which might have contaminated an analytical column, caused a decrease in its separation efficiency, and reduced its lifetime. In order to purify the carbohydrates, TLC was used (20 X 20 cm plates with 0.25 mm thick Silica Gel H). Isopropanol-water-ethyl acetate (54:26:20) was chosen as the developing solvent for TLC. The carbohydrates were eluted with 60 C water from the appropriate TLC bands. Then, the water solutions were concentrated in vacuo. Several other procedures were also used to isolate the carbohydrates: precipitation of proteins with 15% trichloroacetic acid (TCA) (14) and purification of the carbohydrates on preparative columns of hydroxylapatite (HTP), Corasil II, and Corasil/AX).

The products isolated using each of the five methods were then analyzed to determine the carbohydrate composition of the defatted oilseed flours. Three columns packed with polyacrylamide gel with water as the eluting solvent and a flow rate of 1-2 ml/min were tried on the HPLC equipment. The three polyacrylamide gels should separate compounds with molecular weights of up to two, four, and six thousand, respectively. Also a μ BONDAPAK/Carbohydrate column with acetonitrile-water was used at a flow rate of 1.5 ml/min to separate the various carbohydrates. For the cottonseed (glandless and LCP) flours acetonitrile-water (75:25) was selected. A 72:28 ratio was used for the soybean, peanuts, and sunflower flours. The recorded carbohydrate peaks were integrated and their areas were compared to the areas of standard sugars which had been spotted on TLC, developed, and eluted according to the procedure described above.

The amount of total sugars was determined by the phenol-sulfuric acid colorimetric method (2) with dextrose as the standard.

RESULTS AND DISCUSSION

Data in Table I show that although water extracted more protein than the other solvents, the residue from water extraction contained only 0.55% sugar while the residue from the ethanol-water mixtures contained 0.88% and 1.77% sugar. Thus, only 92% of the soluble sugars was extracted with ethanol-water (80:20). This figure was calculated by using the ratio of carbohydrate in the residue to the total carbohydrate in unextracted flour [carbohydrate content was determined according to Dubois (2)]. The differences between the extracting power of water and ethanol-water solvents were more marked for the extraction of oilseed flours which had been pretreated with heat and steam. The residue from the water extraction of heated and steamed flours contained 0.6% carbohydrate. However, the residues from both untreated and pretreated flours of the aqueous alcohol extractions contained at least 1.5% carbohydrate. The carbohydrates remaining in the alcohol extracted residues may contain a larger percentage of higher oligosaccharides than in the flour, which would give erroneous results.

In order to protect the analytical columns and improve the separation, the extracted sugars should be separated as much as possible from the other extracted components. Precipitating the protein with 15% TCA did not totally remove the protein and other components from the sugar fraction. The HTP and Corasil II preparative columns also gave inadequate isolation of the carbohydrate fraction. Of all preparative packing materials tested, Bondapak AX/Corasil gave the best purification of the carbohydrates. However, after ten runs the active sites became saturated and the packing material had to be replaced.

TABLE I

Carbohydrate and Protein Contents of Extracts and Residues from soyflour (% on a moisture-free basis)

Solvent	Extract Protein[a]	Residue Carbohydrate[b]
Water	60.57	0.55
Ethanol-water (60:40)	19.83	0.88
Ethanol-water (80:20)	5.45	1.77

[a]Kjeldahl (15) (Prot. = N x 6.25%)
[b]Dubois, 1956 (2)

The best means of isolating the carbohydrates of defatted oilseed flours was found to be TLC on Silica Gel H plates. With such plates, the composition of the developing solvent was chosen so that the R_f values of all the sugar components were similar. Thus, a separation of the carbohydrates from the other flour components was achieved. A thin layer of Silica Gel H had to be scraped off the plate and the sugars eluted with a small amount of warm water.

Columns packed with polyacrylamide gels and a prepacked BONDAPAK/Carbohydrate column were used to analyze the composition of the isolated carbohydrates. The polyacrylamide packing material designed to identify two thousand molecular weight compounds gave a poor separation of the carbohydrates. The other two polyacrylamide packings for four and six thousand molecular weight separations could not withstand the high pressure and their cross-linkages collapsed. The μ BONDAPAK/Carbohydrate column gave very good resolution of most of the carbohydrates in the defatted oilseed flours and thus was selected as the medium of choice.

Figure 1a shows a typical chromatogram for the carbohydrates of deglanded cottonseed flour (LCP). The solvent peak is a result of the refractive index difference between the eluting solvent and the 65% acetonitrile/water used to dissolve the sugars. The stachyose peak which appears by 24 min is broad. The resolution of this peak could be improved by increasing the polarity of the eluting solvent. Figure 1b shows a chromatogram for the carbohydrates of soybean flour and here stachyose appears after 18 min. However, for the mono- and disaccharides to be adequately resolved in the first part of the chromatogram, a less polar solvent is needed. This may be achieved in a two step analysis. It was found that the first step could start with a less polar solvent (higher percentage of acetonitrile) to give good separation of the mono- and disaccharides. Under such conditions even the traces of the monosaccharides existing in the oilseed flour were separated. In the second step a more polar solvent enabled the larger saccharides to appear as sharp peaks. Another technique would be to keep the solvent ratio constant but to use flow programming. Such a programming system changes the flow rate during the chromatogram. For a better resolution of the mono- and disaccharides a slow flow rate is needed, but towards the end of the run a higher flow rate could be used.

Figures 1c and 1d show the carbohydrate content of the defatted oilseed flours of peanut and sunflower respectively. An unidentified carbohydrate—"D", believed to be a disaccharide (16)—was present in the sunflower.

The nitrogen free extract (NFE) seemed to be more accurate in determining the total carbohydrates (soluble and

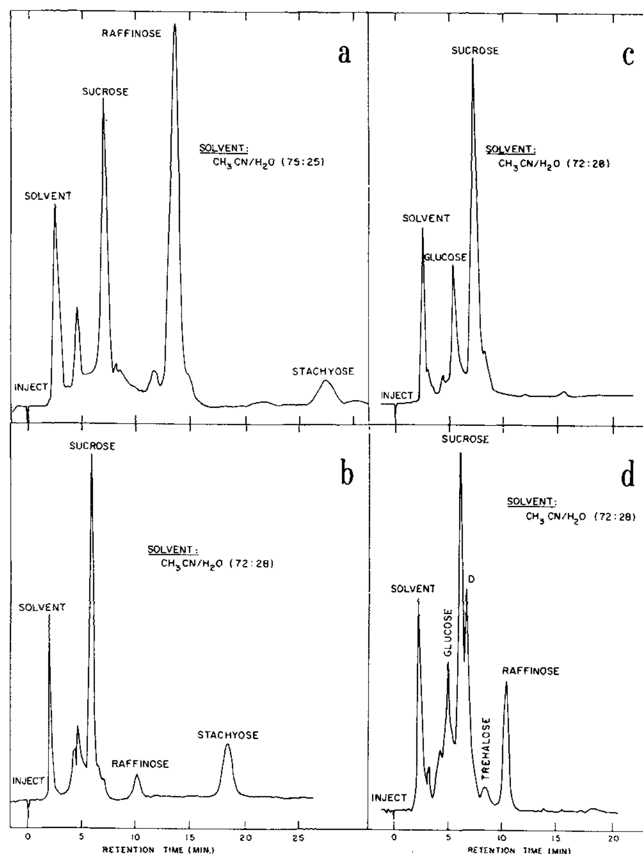

FIG. 1. Carbohydrate chromatograms (high pressure liquid chromatography) using a μ BONDAPAK/column and 1.5 ml/min flow rate of the following defatted oilseed flours: (a) deglanded cottonseed (LCP); (b) soybean; (c) peanut; and (d) sunflower

raffinose, and stachyose. In addition, trehalose, another disaccharide, was found in the flour of sunflower. A difference was noted between the raffinose content between glandless and deglanded cottonseed flour. The liquid cyclone process for removing the toxic glands in the cottonseed is believed to be responsible for the lower content of the raffinose but seems to have almost no influence on the sucrose and stachyose content. The two varieties of peanut flour, Spanish and Virginia, have mainly glucose and sucrose and differ in the content of the latter.

The method described is a simple, versatile and relatively rapid procedure for the separation and determination of the carbohydrate content of oilseed flours using high pressure liquid chromatography. The carbohydrate content of virtually any defatted oilseed flour can be determined with this method. It was shown that without derivatizing the carbohydrates, reproducible, accurate results can be obtained.

TABLE II

Carbohydrate Content[a] of Defatted Oilseed Flours (% on a moisture free basis)

Oilseed flours	NFE	Total	Glucose	Raffinose	Stachyose	Sucrose	Trehalose
Cottonseeds							
Deglanded (LCP)[b]	22.5	9.2	trace	7.93	0.95	2.41	–
Glandless	26.8	16.1	trace	11.95	0.68	2.62	–
Peanuts							
Experimental	–	–	2.34	trace	–	5.52	–
Commercial	22.4	17.4	2.12	trace	–	7.70	–
Soybean	34.0	16.3	trace	1.25	6.30	7.80	–
Sunflower	24.2	8.3	0.60	3.22	–	2.29	0.79

[a]Nitrogen Free Extract (NFE): by subtraction [100 – (Protein + oil + Ash + Crude Fiber)]. Total: by Dubois method 1956 (2). Specific carbohdrates: HPLC method.

[b]Liquid Cyclone Process

insoluble) (17) in oilseed flours than was the Dubois method (2). The total carbohydrates as determined by the phenol sulfuric acid method (2) were found to be as low as 40% of NFE for the deglanded cottonseed flour (LCP) and as high as 78% for the peanut flour. The total soluble carbohydrates (sum of mono- and oligosaccharides as analyzed by the procedure described in the present work) were found to be as low as 26% of the NFE for the flour of sunflower and as high as 56.9% for the glandless cottonseed flour. The data of Table II also indicate that the major carbohydrates in the oilseed flours were glucose, sucrose,

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