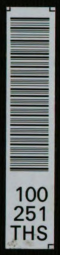


This is to certify that the

thesis entitled

Oligosaccharides of cowpeas

presented by

Maurice A. Akpapunam

has been accepted towards fulfillment
of the requirements for

M.S. degree in Food Science

Pericles Markakis

Major professor

Date February 21, 1978

OLIGOSACCHARIDES OF COWPEAS (VIGNA SINENSIS)

By

Maurice Abiamefune Akpapunam

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Food Science
and Human Nutrition

1978

ABSTRACT

OLIGOSACCHARIDES OF COWPEAS (VIGNA SINENSIS)

By

Maurice Abiamefune Akpapunam

Thirteen cultivars of cowpeas (Vigna sinensis) grown in the Southern United States were first subjected to proximate analysis and then to the identification and quantitative determination of their oligosaccharides.

Paper chromatography supplemented by acid hydrolysis and enzyme hydrolysis showed the presence of sucrose, raffinose, stachyose, and verbascose. Quantitative analysis resulted in the following concentration estimates of these sugars in cowpeas on a dry basis: sucrose 2.2%, raffinose 1.2%, stachyose 3.4%, and verbascose 0.9%.

Sucrose varied considerably between varieties, raffinose was fairly constant, while stachyose and verbascose showed little variation.

The non-digestible oligosaccharides, raffinose, stachyose, and verbascose represented approximately 9% of all sugars present in cowpeas.

ACKNOWLEDGMENTS

The author wishes to express his sincere gratitude to Professor Pericles Markakis, his major professor, for his encouragement and able guidance throughout the course of this work and during the preparation of this manuscript.

Special thanks and appreciation are also expressed to Professor LeRoy Dugan, Jr., Professor Donald H. Dewey, and Dr. Jerry N. Cash for serving in the guidance committee and for reviewing the manuscript and giving helpful suggestions. Dr. Jerry N. Cash was particularly helpful in preparing the slides from which the pictures in this manuscript were made and the author is very grateful to him.

He also wishes to thank Professor Milo B. Tesar of Crop Science Department, Michigan State University, and Professor D. L. Chambliss of Auburn University, Auburn, Alabama, for providing the samples of cowpeas used for this study.

The author is most grateful to the Federal Government of Nigeria for sponsoring his studies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

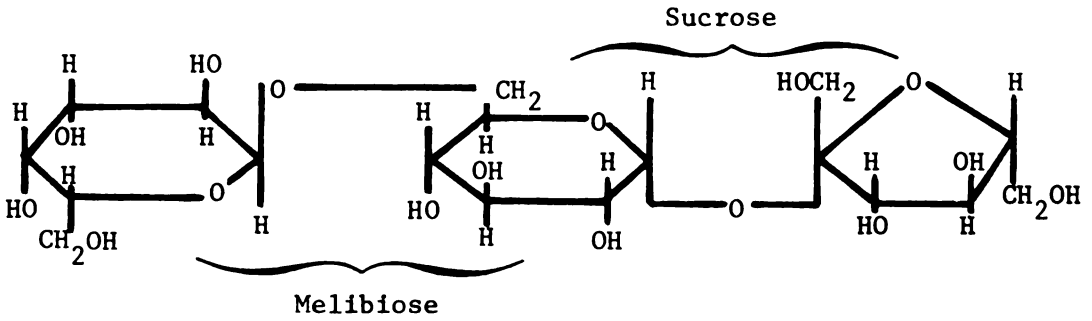
Matured dried beans are staple foods in many countries. In these countries they serve as major sources of protein in the diet. As a result much research had been conducted on the protein quality and quantity of these leguminous seeds. In recent years, the role of the raffinose group of oligosaccharides (raffinose, stachyose, and verbascose) in causing flatulence in man and other animals consuming leguminous seeds has opened up another field of research related to these seeds.

The flatus effect and the discomfort experienced after eating beans and other leguminous seeds are said to be due to lack of α-galactosidase activity in the human digestive tract. The oligosaccharides thus pass into the large intestine where they are fermented by bacteria with the formation of gas and generation of flatus. Relationships between molecular weight of the oligosaccharides and their levels to flatus formation has been reported by Cristofaro et al. (1972). Stachyose and verbascose are reportedly more involved in flatus formation than raffinose.

In nature these oligosaccharides occur freely as soluble reserves in plant tissues or in plant storage organs.

Raffinose is a trisaccharide, consisting of one molecule each of D-galactose, D-glucose and D-fructose. It has been shown

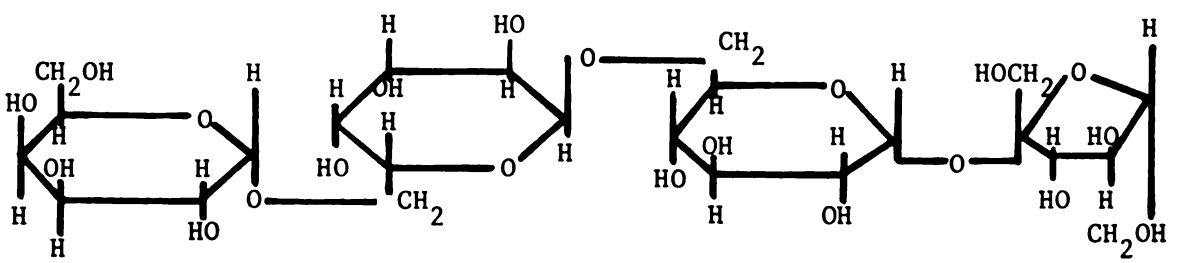
that the fructose and glucose components are joined as in sucrose. The galactose molecule is attached to the glucose with an α (1-6) bond. Partial hydrolysis could yield either melibiose (galactose, glucose) and fructose or sucrose (glucose, fructose) and galactose.



Structure of Raffinose

Raffinose occurs widely distributed in plants, and next to sucrose, it is probably the most abundant oligosaccharide of the plant kingdom. It occurs in sugar beets and is concentrated in sugar beet molasses. Cotton seed contains a good proportion and is generally the material used for its preparation.

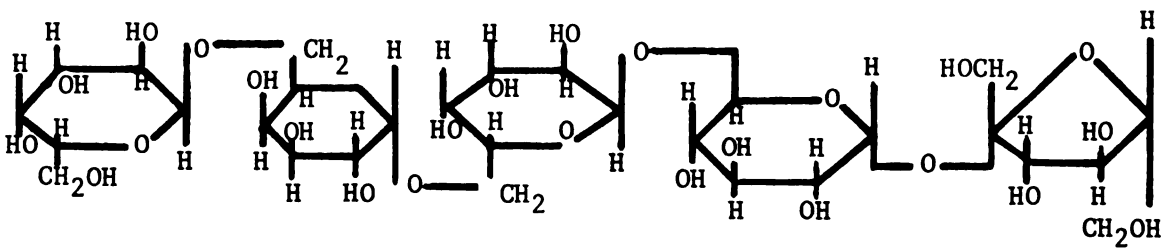
Stachyose is the best known tetrasaccharide. It is composed of two molecules of D-galactose and one each of D-glucose and D-fructose. It is also known as O-α-galactopyranosyl (1 → 6) - O-α-D-galactopyranosyl - (1 → 6) - O-α-D-glucopyranosyl - (1 → 2) - β-D-fructofuranoside.



Structure of Stachyose

On partial hydrolysis with dilute acid, stachyose yields D-fructose, and the trisaccharide, manninotriose. Complete hydrolysis yields D-fructose, D-glucose and two molecules of D-galactose. The tubers of Stachys tuberifera are the classical source of stachyose and may contain up to 75% (dry weight) of stachyose (Bailey, 1965).

Verbascose, like the others is a non-reducing heterogeneous oligosaccharide. It is composed of D-galactose, D-glucose and D-fructose. It is also referred to as O-α-D-galactopyranosly - (1 → 6) - O-α-D-galactopyransyl - (1 → 6) - O-α-D-galactopyranosyl - (1 → 6) - α-D-glucopyranosyl - (1 → 2) - B-D-fructofuranoside. Upon hydrolysis, with dilute acid, D-fructose and a tetrasaccharide, verbascotetraose are formed. This upon further hydrolysis yields three molecules of D-galactose and one of D-glucose.



Structure of Verbasocse

Verbascose occurs in many plants that synthesize sucrose, raffinose, and stachyose, but the most important source is the roots of mullein Verbascum thapsus.

Several workers including Nigam and Giri (1961), Korytnyk and Metzler (1962), Gould and Greenshields (1964), Kim et al. (1973),

Cerning et al. (1975), Tanaka et al. (1975) and others have attempted to separate and quantitate this group of oligosaccharides in various legume seeds. Their findings have so far indicated the presence of these oligosaccharides in relatively large amounts.

This present study was set to investigate the presence of these oligosaccharides in the cowpea. Cowpea or blackeye peas is a tropical legume with its origin in the continent of Africa from where it was introduced to the West Indies, North America, and other tropical and subtropical countries. It belongs to the genus Vigna. There are three well cultivated species of this genus: Vigna sinensis (common cowpea), Vigna unguiculata (catjang), and Vigna sesquipedalis (yardlong bean). Some of the differences between these distinct botanical varieties are given in the table below.

Table 1. The Botanical Varieties of the Cultivated Cowpea[a]

	Seed		Pod	
Botanical Variety	Length	Shape	Length	Shape
Var. sinensis (common cowpea)	6-9 mm	Sub reinform to sub-globose	200-300 mm	Not flabby pendent when mature
Var. unquiculata (catjang)	5-6 mm	Oblong or cylindric	80-130 mm	Not flabby or inflated, erect or ascending when mature
Var. sesquipedalis (yardlong bean)	8-12 mm	Elongated kidney	300-1000 mm	Fleshy and inflated when green, shrink on drying

[a]Table taken from "Tropical Pulses," by J. Smartt (1976).

The seed of this leguminous crop is very nutritious and it is widely consumed in many countries as a pulse and as a vegetable. Because of its high protein content (Table 2), the cowpea has attracted attention as a possible source of protein for the diet of many people in different parts of the world. Several high yielding varieties of this crop have been introduced. Unfortunately this crop like the other members of the legume family contains relatively large amounts of the raffinose group of oligosaccharides.

There have been some attempts to determine the quantity of this group of oligosaccharides in this bean (Nigam and Giri, 1961; Lee et al., 1972). There is, however, no general agreement as to the quantity of these components, in particular, verbascose, in the beans. The discrepancies arose possibly because these components vary with the stage of maturity of the bean seeds (Korytnyk and Metzler, 1962) and perhaps with the variety of the beans. Thus, apart from determining the quantity of these oligosaccharides in the matured, dried, bean seeds, an attempt has been made, in this study, to investigate possible varietal differences among these groups of oligosaccharides in thirteen cultivars of the beans.

LITERATURE REVIEW

Methods Used in Separation and Analysis of Sugars

Recent developments in the chromatographic method of analysis have provided investigators in many fields with an exceedingly powerful and useful tool for separation and identification of sugars in foods and other substances. All the different methods ranging from paper chromatography, paper electrophoresis, thin-layer and column chromatography have been used at one time or another depending on the availability of equipment, time, the extent of investigation and perhaps the amount of material to be analyzed. Paper chromatography is used in identification work. Paper electrophoresis likewise separates sugars for possible qualitative identification or for confirmation. Column chromatography provides a means of obtaining sufficient material for confirmatory chemical studies.

Paper Chromatography

The classical work of Partridge (55, 56), was the first attempt at separation of sugars on paper. Flood, Hirst and Jones (20) placed the paper chromatography of reducing sugars on a quantitative basis using the Somogyi micro-copper reagent. Using hypoiodite oxidation, Hawthorne (28) also determined quantitatively the sugars separated on paper.

Application of paper chromatography for separation of oligosaccharides is similar to that for monosaccharides. Excellent reviews on these determinations are given by Bailey and Pridham (5), Hough and Jones (31), Heftmann (29), and Albon and Gross (1).

Korytnyk and Metzler in 1962 studied the formation of raffinose and stachyose in lima beans. They utilized paper chromatography for the separation and identification of the sugars. Nigam and Giri (49) employed paper chromatography for the separation and quantitation of oligosaccharides present in eight pulses.

Bailey and Pridham (6) and Bailey (4) have outlined a number of procedures for the structural identification of oligosaccharides.

In recent years colorimetric methods have achieved popularity over the classical analytical methods which utilize the reducing properties of sugars to determine their quantities gravimetrically or titrimetrically. This is because they are simple and are applicable to very small amounts of materials such as are separated by chromatographic procedures. Volumetric procedures involving the use of potassium ferricyanide, ceric sulfate, copper sulfate, and sodium hypoiodite are applicable to the determination of small amounts of reducing sugars after separation by partition chromatography. However, experience shows that these methods require considerable skill, they are time consuming and sensitive to slight variations in the conditions.

The anthrone and 1-naphtholsulfate colorimetric reagents are excellent for standard sugar solutions, but when applied to the analysis of sugars separated by partition chromatography, the presence

of only traces of residual solvent developer have been found to render them useless. Residual phenol, for instance, holds tenaciously to the filter paper, interfering with the green color produced by the anthrone reagent. Another disadvantage of the anthrone reagent is the high cost and the fact that its solution in sulfuric acid is not stable. The method also suffers from the disadvantage that, while it is satisfactory for free sugars and their glycosides, it is of limited use for methylated sugars and the pentosans. Residual propionic acid is also known to interfere with the 1-naphthol sulfate reagent. This limits its use for determination of disaccharides for which butanol-propionic acid-water is an excellent solvent for separation.

Aniline phthalate and aniline trichloroacetate have been utilized for the colorimetric determination of sugars and their derivatives; the reagents have, however, proved unsatisfactory for ketoses.

The phenol-sulfuric acid method of Dubois et al. (7) has proved to be very useful for the quantitative determination of simple sugars, oligosaccharides, polysaccharides, and their derivatives. The method is particularly useful for the determination of small quantities of sugars separated by paper partition chromatography with the phenol-water solvent and also for the sugars separated with solvents which are volatile; for example, butanol-ethanol-water, ethylacetate-acetic acid-water, or methylethyl-ketone-water. The reagents are stable, also the color produced is stable and insensitive to changes in conditions.

A number of reagents have been utilized for detection of sugar spots. Among these is ammoniacal silver nitrate (55) which is said to be universal in application. It, however, has some disadvantages; for example, it is known to react with a very wide range of reducing substances other than the sugars. Also sugar spots may migrate from the wet spot to the dry area of the filter paper. Another disadvantage of the method is the need for controlled heating. To overcome some of these disadvantages, Trevelyan et al. (74) introduced a method which eliminates the heating step. Also in this method, reagents are applied in organic solvents, thus reducing the danger of migrating spots.

Triphenyltetrazolium chloride (47) can be used as a spray reagent for sugars. It is, however, time consuming and it is not good for non-reducing sugars. A solution of benzidine in glacial acetic acid has been used by Horrocks (30) as a chromatographic spray reagent for detection of sugar spots. A modification of this method involving the use of a mixture of benzidine in glacial acetic acid and trichloroacetic acid in water (67) is very suitable for both reducing and non-reducing sugars.

Other spray reagents include aniline hydrogen phthalate (57), α-naphthol-phosphate (1), naphthoresorcinol-trichloroacetic acid (56), and dinitrosalicylate reagent (78). While most of these reagents have been used for monosaccharides, only very few are applicable to oligosaccharides and polysaccharides.

Paper Electrophoresis

This technique is used for separating charged materials using a support, buffers, and an electric current. The manipulations are in many ways similar to paper chromatography except for the actual development of the chromatograms in the migration chamber. The technique can be used to provide useful confirmatory evidences regarding the structure of an oligosaccharide. In fact, it is suggested that wherever possible, electrophoresis should be used in conjunction with paper chromatography and the identity of an oligosaccharide checked by comparing its electrophoretic rate of movement with that of an authentic specimen.

Several papers and reviews have been presented on paper electrophoresis; details can be obtained from Bailey and Pridham (5), Foster (18, 19), and Block et al. (9). Most observations on the electrophoretic behavior of oligosaccharides have been carried out using an alkaline borate buffer. Foster (19) recorded the rates of movement, relative to D-glucose (MG values), of various glucopyranosyl disaccharides, and Bailey and Pridham (5) have examined the mobilities of the raffinose family of oligosaccharides and a series of 1-4-β-linked D-mannose oligosaccharides (D.P. 2-5). In the case of the raffinose series, the behavior is highly characteristic of the group, the mobilities increasing in the order; raffinose < stachyose < verbascose < tetragalactosyl-sucrose. (This is the reverse order of that observed in paper chromatography.) With mannose oligosaccharides, however, the more usual behavior of decreasing rate of movement with increasing molecular weight was observed.

Some other electrolyte systems which have been used for this technique include sodium arsenite, basic lead acetate, sodium hydroxide, sodium tungstate, sodium-metavanadate, and ammonium or sodium molybdate. With the exception of ammonium molybdate all the other electrolytes were applied mainly to monosaccharides.

Gas-Liquid Partition Chromatography

Separation of carbohydrate derivatives by gas-chromatography is quite recent. The delay probably might be due to some restrictions of the method. For example, the material subjected to gas-chromatography must be volatile, stable to the operating conditions of the instrument, and not irreversibly adsorbed on the chromatographic column. Methyl glycosides of monosaccharides fulfilled these requirements and were the first group of carbohydrate derivatives investigated. Using programmed temperatures for the separation, oligosaccharide esters have also been separated and analyzed by this technique.

The gas-chromatographic technique has the advantage of speed, and the fact that the fractions can be analyzed quantitatively and collected for identification is another added advantage. The equipment is, however, very expensive. Detailed reviews on its use for carbohydrates are given by Kircher (41) and Bishop (8).

Thin-Layer Chromatography

The first attempts to separate sugar mixtures by thin-layer chromatography were made in 1961 (70, 69, 54). Since then many papers concerning this problem have continued to appear. Poor separation of

some of the more common sugars and the low capacity of the chromatoplates are two disadvantages of this technique.

In most earlier reports, silica gel G, kieselguhr, gypsum, cellulose powder, as well as silica gel and/or kieselguhr impregnated with boric acid, sodium tetraborate, bisulfulite and acetate have been used on the chromoplates. Recently thin-layer chromatography on silica gel impregnated with sodium hydrogen phosphate or sodium monohydrogen phosphate in phosphoric acid was successfully employed by Ovodov et al. (52). They claimed the method is quite suitable for mono, oligo, and polysaccharides.

Tanaka et al. (72) developed a procedure using thin-layer chromatography and thiobarbituric acid reagent for the quantitative determination of oligosaccharides in legume seeds. They reported excellent recovery.

Cerning et al. in 1975, also utilized thin-layer chromatography in their studies of the carbohydrate composition of horse beans. Excellent results were reported.

Column Chromatography

Column chromatography is fast becoming an important tool for separation of carbohydrates. A good account of this technique is given by Binkley and Wolfrom (7). Many materials have been used as the stationary phase for columns. Among these are charcoal, cellulose powder, starch, celite, clay, silica gels, sephadex, and ion-exchange materials.

Separation of oligosaccharides on column was reviewed by Bailey and Pridham (5). According to them, the separation can be effected by three main methods:

1. Partition chromatography on cellulose or celite,
2. Adsorption chromatography on charcoal, and
3. Ion-exchange resin chromatography.

Newly developed column packings for high performance liquid chromatography has greatly simplified the separation of sugars in food substances. Palmer in 1975 described a simple, rapid and versatile method for the separation and determination of sugars via high pressure liquid chromatography. He claimed that virtually any combination of monosaccharides, disaccharides and/or higher oligomers can be separated by this method. Havel et al. (27) determined the oligosaccharides released during hydration of textured soy with the aid of high performance liquid chromatography.

Floridi in 1974 applied automatic ion-exchange chromatography for simultaneous determination of sugars and sugar alcohols in foods.

Specific Enzyme Methods

A number of methods have been described which utilize enzymes for the determination of carbohydrates in biological fluids, such as, blood and urine (15, 16, 62). Many of these methods are presently employed for the determination of carbohydrates in foods (32, 76, 39).

D-glucose, for example, can be determined in a mixture of sugars with a coupled enzyme system containing glucose oxidase or

more properly, β-D-glucose: oxygen oxido-reductase. The principle behind the reaction is summarized below:

D-glucose $\xrightarrow[\text{oxidase}]{\text{glucose}}$ D-gluconic acid lactone + hydrogen peroxide

Hydrogen peroxide $\xrightarrow{\text{catalase}}$ water + oxygen

Oxygen + reduced leuko-chromogen $\longrightarrow$ oxidized colored chromogen

A test kit, glucostat, is commercially available, for quantitative colorimetric analysis. The kit contains buffer, reduced chromogen, catalase, and glucose oxidase.

D-fructose has been determined in mixtures containing only glucose or sucrose by differences in reducing power before and after oxidation of the glucose with glucose oxidase (79).

D-galactose may be determined quantitatively in mixtures of monosaccharides by a coupled enzyme system containing galactose oxidase in a way similar to that used for D-glucose. The enzyme is known to be active on free D-galactose as well as D-galactopyranosyl residues of oligo and polysaccharides. In fact, Avigad et al. (2) found that the tetrasaccharide, stachyose, was oxidized six times faster than D-galactose. It is evident that this enzyme is useful for the quantitative analysis of oligosaccharides.

Rot et al. (62) defined certain conditions which will permit adaptation of the galactose oxidase reaction to a generally useful method for the determination of galactose in a variety of situations. These include, that the sample should be at approximately neutral pH,

and free of the interfering substances enumerated below: protein precipitants and other substances capable of injuring enzymes in general (e.g., large amounts of ethanol) should be obviously eliminated. One further condition which has been found to affect the galactose oxidase reaction adversely appears to be high ionic strength or osmolarity. Thus, high concentrations (exceeding approximately 0.5 M) of simple salts: chlorides, sulfates, and phosphates of sodium or potassium and organic compounds (glucose, glycine, tris, succinate, sucrose) have been found to inhibit the enzyme.

Oligosaccharides of Legumes

Legume seeds are particularly rich in oligosaccharides of the galactosyl sucrose type (raffinose, stachyose, and verbascose).

Nigam and Giri (49) examined the seeds of some pulses including cowpea (Vigna sinensis) for their oligosaccharide composition. They found the chromatographic pattern of all the pulses examined to be the same. The carbohydrates were non-reducing and yielded glucose, galactose, and fructose on hydrolysis. The oligosaccharides were confirmed as raffinose, stachyose, and verbascose. The determination of each of these component sugars in the various pulses showed uniformity. Each one contained a large amount of verbascose followed by stachyose and then sucrose, with relatively small amounts of raffinose. They stated that this raffinose family of oligosaccharides seemed quantitatively more important than the polysaccharides which occur in the pulses in a much lower amount.

Nigam and Giri also observed that soaking the leguminous seeds in water brought about a reduction in the amount of these oligosaccharides in the seeds. After about 48 hours soaking, the raffinose and stachyose content completely disappeared. Sucrose was reported constant during the soaking and the germination that followed. Fructose was observed to increase with time. They could not strike any balance between the disappearance of the ologosaccharides and the formation of sucrose and the monosaccharides. They regarded the phenomenon as a result of α-galactosidase activity of the germinating seeds which hydrolyzes the oligosaccharides to monosaccharides and sucrose. Glucose, fructose, and to a large extent galactose play an important function in plant growth and the synthesis of cellulose and stem protein.

Gomyo and Nakamura (24) confirmed the presence of raffinose in legumes, stating that it could be synthesized from uridine diphosphate, galactose, and sucrose. They also observed that in seeds raffinose appears during maturation and disappears during germination, a view also maintained by Gould and Greenshields (25), and Nigam and Giri (49).

Cerning et al. (12) reported varying amounts of verbascose, stachyose, raffinose, sucrose, and in some cases, traces of glucose and fructose in horse beans. They obtained an average value of 5.7% for these ethanol soluble sugars in these beans.

Korytnyk and Metzler (42) studied the formation of raffinose and stachyose in lima beans. They observed that a decrease in stachyose

was accompanied by a corresponding increase in fructose, whereas the relative amount of sucrose and raffinose remained fairly constant. They suggested a stepwise formation of the sugars. Sucrose may be formed from fructose and uridine diphosphate glucose, whereas, at a later stage of development, raffinose is formed from sucrose and a corresponding galactose intermediate. Similarly, stachyose could be formed from raffinose by a transgalactosylation reaction.

Pritchard et al. (58) indicated that the composition of the ethanol soluble fractions of winter field beans varied considerably between varieties. They maintained that the main components were oligosaccharides and sucrose or its hydrolysis products.

Tanaka et al. (72) determined the oligosaccharides of soybeans, mungbeans, adazuki beans, and white beans. They did not report the presence of verbascose, but their value for raffinose, stachyose, and sucrose agreed fairly well with those of other workers.

Soybeans seem to have a higher percentage of sucrose than most other legumes. The values obtained by Kawamura (37), showed sucrose highest followed by stachyose and then raffinose. This sequence agrees with the findings of Tanaka et al. (72) and the data of Hardinge et al. (26).

Studies on soybeans have shown that the raffinose group of oligosaccharides tend to disappear after germination and soaking or autoclaving (40, 38, 80). Germination and irradiation of navy beans also reduce the oligosaccharides content (68). One may, therefore, consider that soaking and/or blanching as well as germination and

irradiation of leguminous seeds might well serve as a method for the removal of oligosaccharides from these seeds.

The above reviews have all indicated the presence, in beans, of the raffinose group of oligosaccharides. There is, however, no general agreement about the quantities of each sugar in the various legumes. The variations in the reported values could be as a result of the different methods of analysis. It could also have been due to the stage of maturity of these beans as well as the fact that these sugars vary with the variety of the beans.

MATERIALS AND METHODS

Preparation of Samples

Thirteen different varieties of dried, matured cowpeas (Vigna sinensis) grown in the Southern United States were used for this study.

A 10 gram sample of the well-ground seeds from each of the varieties was covered with 70% cold ethyl alcohol and extracted with constant agitation for 12 hours. The resulting mixture was then filtered through a Buchner funnel. The residue was washed three to four times with small portions of cold 70% alcohol. The combined extracts were then concentrated under vacuum at about 40°C. The concentrated syrup was clear and needed no further clarification. It was made up to 25 ml with distilled water and stored in the refrigerator (0-5°C).

Attempts at extraction with 80% hot ethanol produced a syrup containing oil and some slimy materials which could not be removed by centrifugation.

Separation and Identification of the Oligosaccharides

The sugars were separated by paper chromatography using Whatman No. 1 filter paper (46 x 57 cm) with serrated lower edge to prevent irregular solvent flow.

Known volumes of the sample extracts were spotted on a line 7.5 cm from the top edge of the filter paper. Standard sugars: sucrose, glucose, fructose, stachyose, raffinose, galactose, and melibiose were also applied at 2.5 cm intervals along the same line. The standards had a concentration of 1% (w/v) in 10% (v/v) 1-propyl alcohol. To avoid obtaining wide spots and consequent spreading, each spot was dried by a current of hot air from an electric hair dryer after the application of about 1 λ (1 µl, 0.001 ml) from a microsyringe. The paper was then left to air dry for about 30 minutes. Next, the paper was placed in a chamber which had been saturated with the developing solvent. After about an hour, the developing solvent was added through a small hole on top of the chamber cover onto a trough holding the paper.

The overrun development method was employed because of the very low R_f values of the oligosaccharides. The development lasted for 48 hours.

The following solvent systems were tried:

A. n-butanol, ethanol, water (5:3:2 by volume);

B. n-butanol, ethanol, water (10:1:2 by volume);

C. 1-butanol, pyridine, water (6:4:3 by volume); and

D. 1-butanol, acetic acid, water (4:1:5 by volume).

Of these, the first and third solvents (A and C) produced good separation. Solvent A, however, was preferred to Solvent C because of the irritating odor of the latter. Both are monophasic mixtures and therefore easy to work with, since one does not have to worry about their eventual separation.

At the end of the development, the paper was brought out and dried in air for about an hour. It was then sprayed with benzidine reagent to detect the sugar positions. The paper was allowed to dry in air and then heated in an oven at 100°C to 105°C for 5-10 minutes.

The benzidine spray reagent was prepared from two solutions:

- 1 g benzidine in 40 ml glacial acetic acid--Solution A; and
- 30 g of trichloroacetic acid in 40 ml of water--Solution B.

These solutions were mixed before use and diluted with about 10 ml of acetone. The reagent reacts with almost all sugars producing intense dark brown coloration with most of them and yellow with fructose. Other reagents tried include α-naphthol-phosphoric acid for detecting ketoses and triphenyl-tetrazolium chloride for reducing sugars.

Characterization of the Oligosaccharides

Approximately 100-200 µl of sugar extracts were applied to the filter paper by transverse streaking. Several sheets of paper were streaked with the extract in order to obtain sufficient quantities of individual sugars after elution. The portion occupied by the sugar was eluted from the papers following the procedure described below for quantitative analysis. The eluates of each individual sugar were combined, concentrated, and subjected to the following tests.

1. Test for Reducing Sugars. Ten ml of the eluted sugar was made alkaline with 1N NaOH solution in a test tube. The tube was then placed in a water bath at 25°C. After about 2 minutes, 2 ml of 0.5% triphenyltetrazolium chloride was added to the tube. The mixture,

properly shaken, was left in the bath for exactly 30 minutes. Formation of a red precipitate after that time indicates presence of reducing sugars.

2. Test for Presence of Fructose in the Sugar Structure. The separated sugar spots on the paper were sprayed with α-naphthol-phosphate reagent (1). Purple coloration of the spots indicates the presence of fructose in the structure of the sugars.

3. Raybin's Test (for Sucrose and Sucrose Containing Compounds). Five ml of the eluted sugar in 5 ml of N/20 NaOH (at 10°C) was shaken in a closed test tube with 7-10 mg of diazouracil until the latter dissolved. A blue-green color developed within a few minutes. The color faded, turning brownish on standing at room temperature. The blue-green color was given by all the cowpea sugars.

4. Hydrolysis of the Oligosaccharides. Some quantities of the eluted sugar solution were hydrolyzed with 1N H_2SO_4, for 2 hours at 100°C. The resulting hydrolysates were then rechromatographed using n-butanol, ethanol, water (5:3:2 by volume) on Whatman No. 1 filter paper. Enzymic hydrolysis with α-galactosidase and invertase were also attempted.

5. Correlation of Structure with Papergram Mobility. This test was performed with the original sample extract. Five to ten microliters of the extract together with standard sugars (sucrose, raffinose, and stachyose) were spotted on two filter papers. The papers were developed along one direction (unidimensional) with n-butanol, ethanol, water (5:3:2 v/v) and 1-butanol, pyridine, water

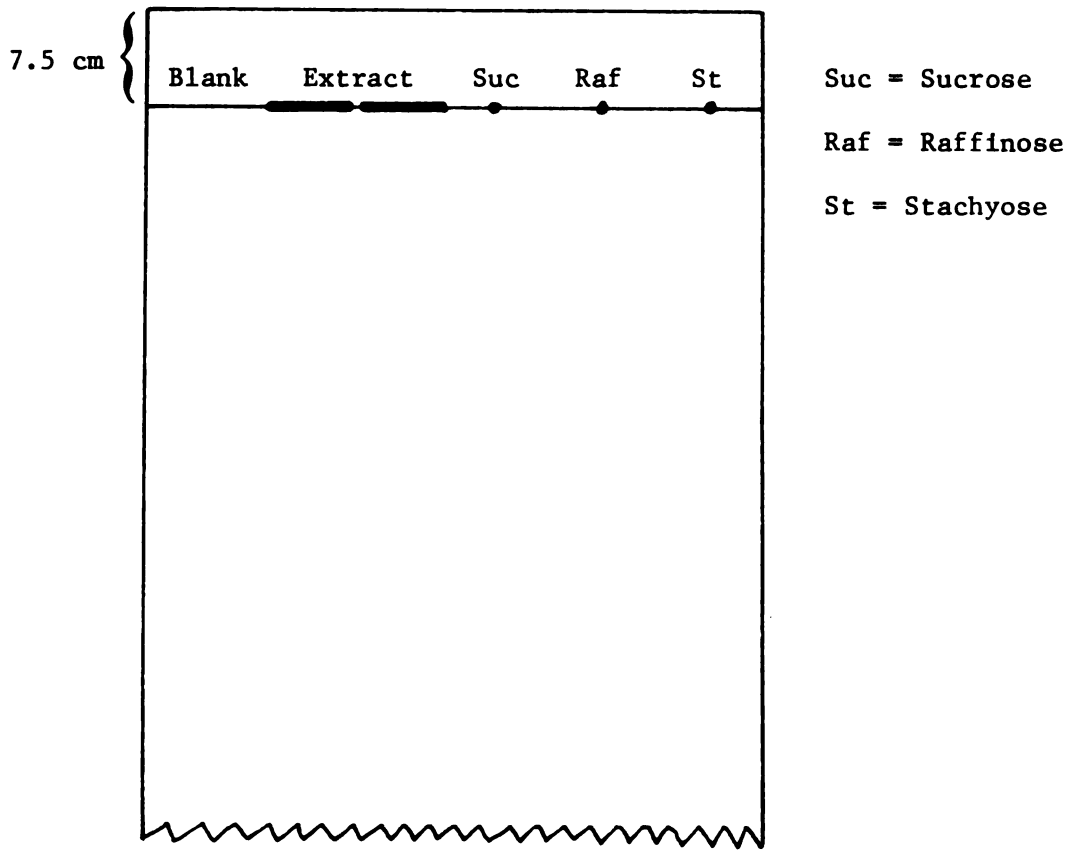
(6:4:3 v/v), respectively. The sugar spots were identified with the benzidine spray reagent. The R_f values of the individual sugar components from the two chromatograms were determined and from this the partition function, α, (log α = Log $[R_f/(1-R_f)]$ was calculated. Log α was then plotted against the number of hexose units (molecular size) in each component.

The above procedure was repeated for the glucose terminal series of sugars obtained by invertase hydrolysis of the oligosaccharides of the cowpeas. The separation on the filter paper was with n-butanol, ethanol, water (5:3:2 v/v) solvent system.

Quantitative Analysis

Two methods were used: the phenol-sulfuric acid colorimetric method (17) and the modified resorcinol-hydrochloric acid method (61). The former was applied to the determination of sucrose, raffinose, and stachyose and the latter to the determination of verbascose.

The sugars were first separated on Whatman No. 1 filter paper and then eluted for the analysis. As shown on the following sketch, at a distance of 7.5 cm from the straight edge of a 46 x 57 cm Whatman No. 1 paper one linear portion (5 cm) was reserved as a blank space, a second portion (31 cm) was streaked with the cowpea extract and a third portion (10 cm) was spotted with authentic sucrose, raffinose, and stachyose solutions. The streaking of the extract was done in duplicate. Each portion, 15 cm long, contained 50 µl of the extract. The paper was then developed as in the qualitative analysis using Solvent A.



Sketch of filter paper set up.

After drying, the strip containing the standard sugars was cut and sprayed with the α-naphthol-phosphoric acid reagent, to locate the sugar positions. The strip was then matched against the main paper and portions corresponding to each sugar was cut out from the main paper. Each section containing the sugar was further cut into smaller pieces and placed in a beaker containing 10 ml of deionized water. The beaker was then left covered for 30 minutes, with occasional shaking. A portion was cut from the blank space corresponding to the sugar portions and treated as above. The extracted sugars were then filtered through

sintered glass wool and collected in test tubes with covers. One ml of each of these was then pipetted into other test tubes. To this was added 1 ml of 5% phenol reagent. After mixing, 5 ml of concentrated sulfuric acid was added quickly to each of the tubes. The mixture was shaken to mix properly and then cooled to room temperature, using running water from the tap. The absorbance of the resulting yellow-orange color was determined at 490 nm with a Beckman model DU Spectrophotometer.

Standard curves were prepared for each sugar using concentrations in the range of 10-70 μg per ml. The blank absorbance value was subtracted from the absorbance of each of the sample sugars before reading off the concentration from the standard curve.

The above method could not be applied for the determination of verbascose, because no authentic verbascose was available to prepare a standard curve. Instead the eluted sugar was partially hydrolyzed with yeast invertase (also with 0.05 N HCl) to liberate fructose which was determined with Roe's reagent (61). To 1 or 2 ml of the hydrolysate was added 2 ml of 0.1% alcohol resorcinol followed by 6 ml of 30% HCl. The tube was heated in a water bath at 80°C for eight minutes. The purple color obtained was measured with a spectrophotometer at 520 nm.

A standard curve was prepared using fructose ranging from 0.025 to 0.1 mg. The obtained fructose values were converted to verbascose by multiplying by a factor of 4.6.

Determination of "Total Carbohydrate"

The percentage of total carbohydrate content of the cowpeas was obtained by subtracting from 100 the percentage of contents in moisture, ash, protein (N x 6.25), fiber, and lipid. All the components with the exception of fiber were determined by the methods outlined in the AOAC (50) methods of analysis. The fiber was determined by the modified trichloroacetic acid digestion reagent method of Whitehouse et al. (77).

RESULTS AND DISCUSSION

Figure 1 is a display of the thirteen cowpea cultivars used for this study. They all belong to Vigna sinensis. The color and size of the seeds varied among cultivars. The color ranged from creamy-white to dark brown. The pinkish color of SA Dandy variety is a result of pretreatment against infestation. The true color is creamy-white.

The composition of the cowpeas is given in Table 2. It shows that the seeds are high in protein and carbohydrate, low in oil and fairly high in crude fiber and ash. The protein, oil, ash, and carbohydrate showed some uniformity in all the varieties while the crude fiber varied to some extent. All the values are the means of two determinations, which varied by less than 4%.

Separation and Identification of the Oligosaccharides

Figure 2 shows the separation of the oligosaccharides of some of the cowpea varieties on paper. The chromatographic pattern of all the varieties examined were found to be the same. The chromatogram showed that there are four types of oligosaccharides in these cowpea varieties. The R_G and R_f values of authentic sugars and of the sugars of cowpeas with two different solvents are shown in Table 3. The solvents were: n-butanol, ethanol, water, 5:3:2 by volume, and 1-butanol, pyridine, water, 6:4:3 by volume. These data indicate

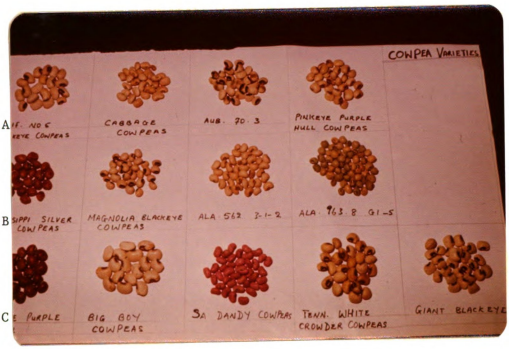


Figure 1. Thirteen cultivars of cowpeas (Vigna sinensis) used for this study. (Sa Dandy cowpeas true color is creamy-white; pink color is the result of pretreatment.)

Note: A = California No. 5 Blackeye; B = Mississippi Silver Skin; C = Knuckle Purple Hull.

Table 2. Proximate Composition of 13 Cowpea Cultivars (g/100 g sample as received)[a]

	Cultivars												
	California No. 5	Cabbage Cowpea	Auburn 70.3	Pinkeye Purple Hull	Mississippi Silver Skin	Magnolia Blackeye	Alabama 963.8 G1-5	Alabama 562 3-1-2	Knuckle Purple Hull	Big Boy Cowpea	Sa Dandy Cowpea	Tenn. White-crowder	Giant Blackeye
Moisture	7.9	9.1	8.4	10.1	10.1	9.9	9.0	10.9	10.6	8.3	10.1	11.7	8.9
Protein (N x 6.25)	26.3	26.3	24.2	24.5	23.6	21.0	22.8	22.8	24.5	24.5	23.5	22.7	24.5
Fiber	2.2	2.4	4.4	3.4	4.6	5.2	5.1	3.3	3.6	4.1	5.9	2.9	5.7
Ash	3.0	3.4	3.2	3.5	3.3	3.5	3.3	3.2	3.1	3.4	3.5	3.3	3.7
Lipids	1.3	1.3	1.8	1.9	1.7	1.7	1.5	1.3	1.8	1.8	1.5	2.0	1.3
Carbohydrates (by difference)	59.3	57.5	58.0	56.6	56.7	58.7	58.3	58.5	56.4	57.9	55.5	57.4	59.9

[a]Average of two determinations differing by less than 4%.

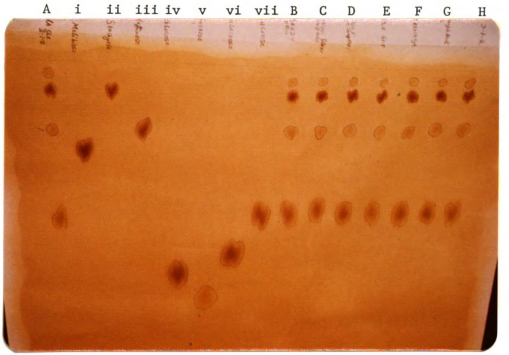


Figure 2. Descending paper chromatography of standards and cowpea oligosaccharides.

Solvent: n-butanol, ethanol, water, 5:3:2 by volume.
Standards: i, melibiose; ii, stachyose; iii, raffinose; iv, glucose; v, fructose; vi, galactose; vii, sucrose.
Varieties: B = Sa Dandy; C = Mississippi Silver Skin; D = California No. 5; E = Alabama 963.8 GI-5; F = Giant Blackeye; G = Pinkeye Purple Hull; A and H = Alabama 562 3-1-2.

Table 3. R_G and R_f Values of Authentic Sugars and Cowpea Sugars

	R_G Values		R_f Values			
	(B-E-W)[a]		(B-E-W)[a]		(B-P-W)[b]	
	Authentic Sugars	Cowpea Sugars	Authentic Sugars	Cowpea Sugars	Authentic Sugars	Cowpea Sugars
Sucrose	.69	.68	.24	.24	.23	.24
Raffinose	.30	.28	.11	.11	.11	.11
Stachyose	.13	.12	.05	.04	.04	.04
Verbascose	--	.06	--	.02	.02	.02

[a]B-E-W: n-butanol, ethanol, water, 5:3:2 by volume.

[b]B-P-W: 1-butanol, pyridine, water, 6:4:3 by volume.

the presence of sucrose, raffinose, stachyose and probably a pentasaccharide in cowpeas. Figures 3 and 4 are sketches of the separation of these sugars on paper with the two solvent systems.

Increasing the separation time from 48 hours to 60 hours did not show any spot between origin and the pentasaccharide. Increasing the spotted quantity of extract did not result in the appearance of more spots on the chromagtogram.

When the separation was first carried out with the fresh extracts there were no spots indicating the presence of glucose or fructose (Figure 2). When the extracts were kept in the refrigerator (0-5 C) and then analyzed after five months in a way identical to that for the fresh extracts, two more spots appeared on the chromatogram.

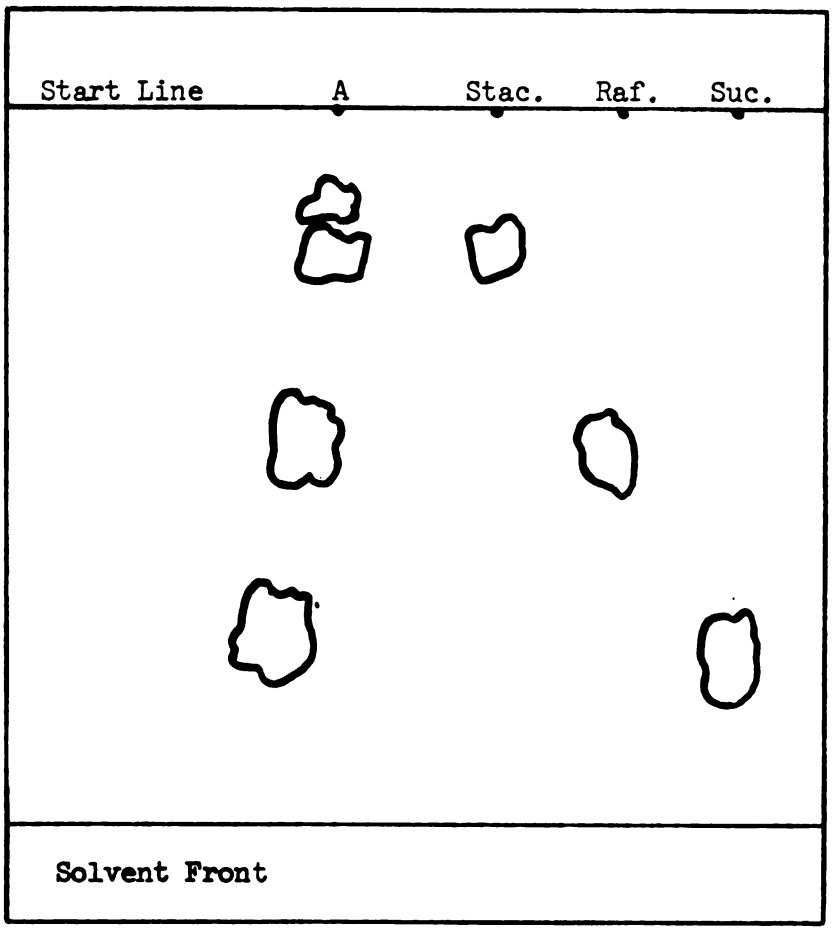


Figure 3. Descending paper chromatography of standards and cowpea oligosaccharides.

Solvent: n-butanol, ethanol, water (5:3:2 v/v).
Standards: Stac., Stachyose; Raf., Raffinose; Suc., Sucrose; A = Giant Blackeye Cowpea.

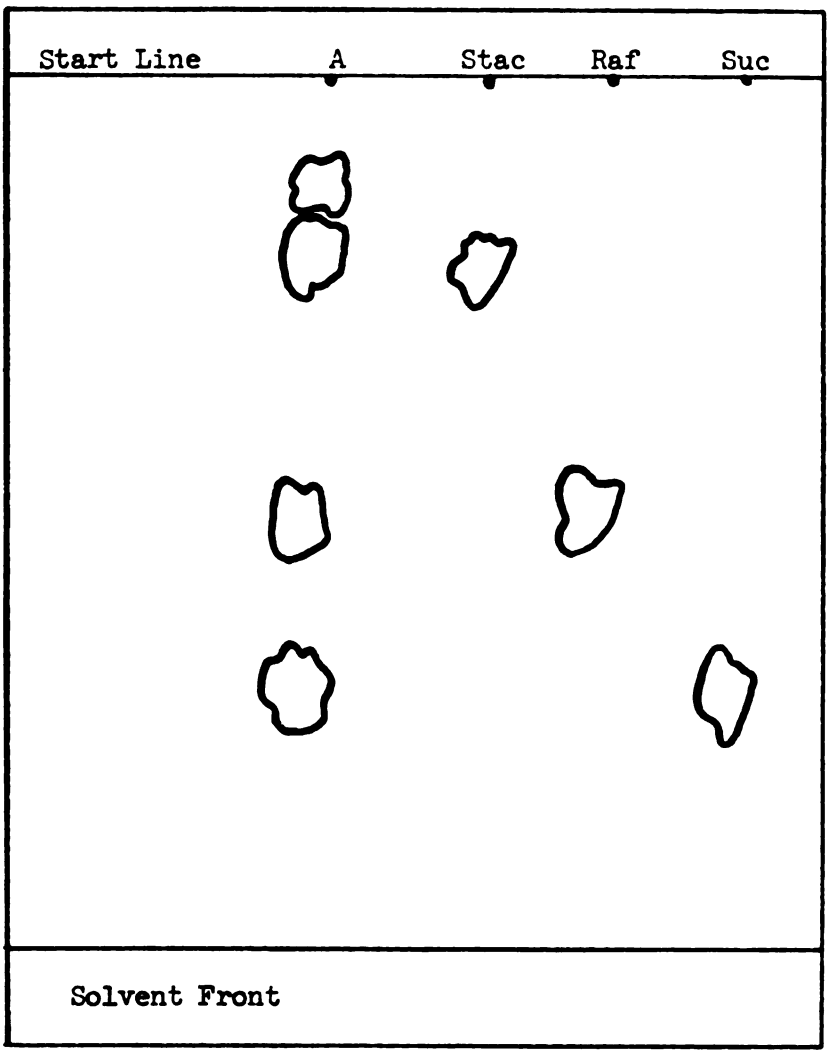


Figure 4. Descending paper chromatography of standards and cowpea oligosaccharides.

Solvent: 1-butanol, pyridine, water (6:4:3 v/v).
Standards: Stac., Stachyose; Raf., Raffinose; Suc., Sucrose; A = Giant Blackeye Cowpea.

Cerning et al. (12) reported similar findings with horse beans. The monosaccharides were probably formed by hydrolysis of the oligosaccharides through glycosidases extracted along with the sugars.

Further Identification and Confirmation of Individual Sugars

1. Test for Reducing Sugar. The sugars did not give any color when treated with triphenyltetrazolium chloride reagent. This is an indication that they are non-reducing (49, 47).

2. Test for Presence of Fructose and Sucrose in the Sugars. When the sugars were treated with α-naphtylamine-phosphate (1), a purple color was produced. This is a positive test confirming the presence of fructose in the sugars. Further structural evidence was given by the diazouracil test (60). The blue-green color given by all the sugars was a positive indication of the presence of sucrose in the structures of these sugars.

3. Hydrolysis of the Sugars. Partial hydrolysis, with dilute mineral acid and yeast invertase gave fructose, glucose, melibiose, a trisaccharide, and a tetrasaccharide (Figure 5). Complete hydrolysis with stronger mineral acids gave fructose, glucose, and galactose only (Figure 6). All these findings are consistent with the structure of the oligosaccharides, sucrose, raffinose, stachyose, and verbascose.

4. Correlation of Structure with Papergram Mobility. In a homologous series of sugars a linear relationship exists between Log $[R_f/(1-R_f)]$ (partition function), and the number of hexose units in

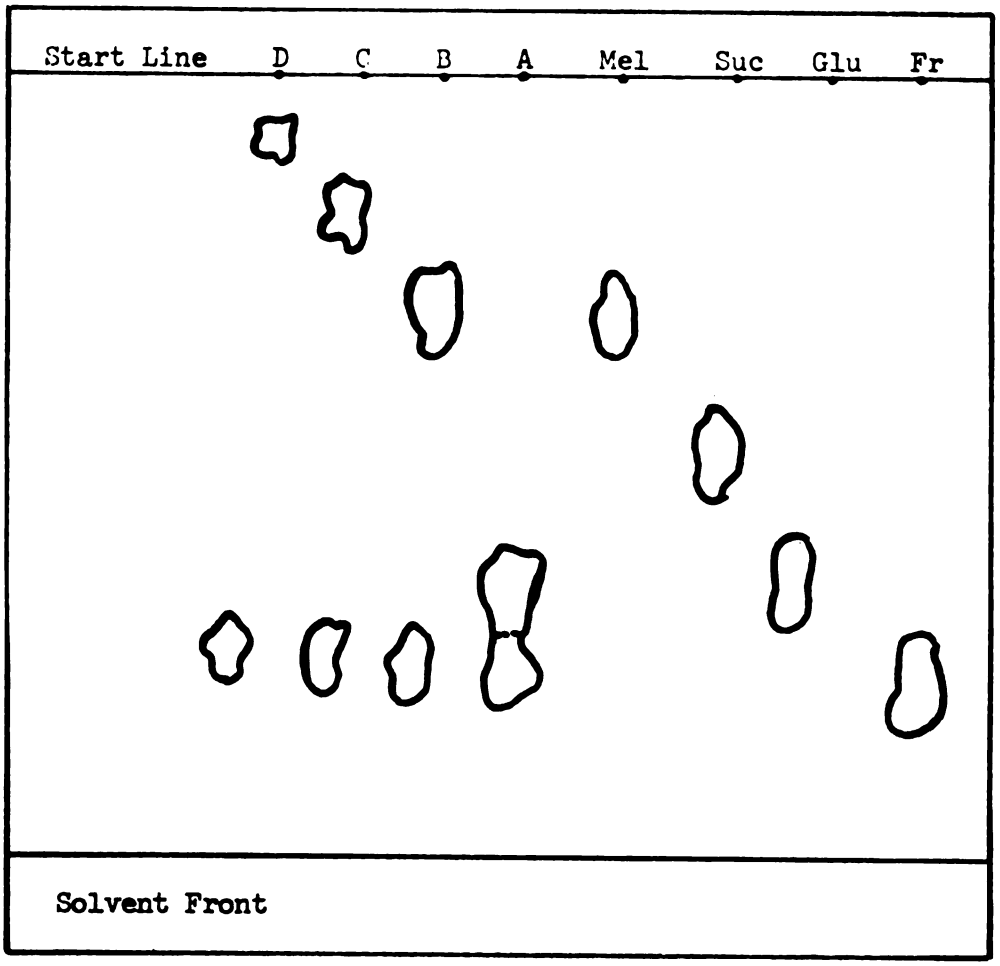


Figure 5. Descending paper chromatography of the product of invertase action on sugars of cowpeas.

Solvent: n-butanol, ethanol, water (5:3:2 by volume). Mel., Melibiose, Suc., Sucrose; Glu., Glucose; Fr., Fructose; Fr., Fructose; A = Sucrose hydrolysate; B = Raffinose hydrolysate; C = Stachyose hydrolysate; D = Verbascose hydrolysate.

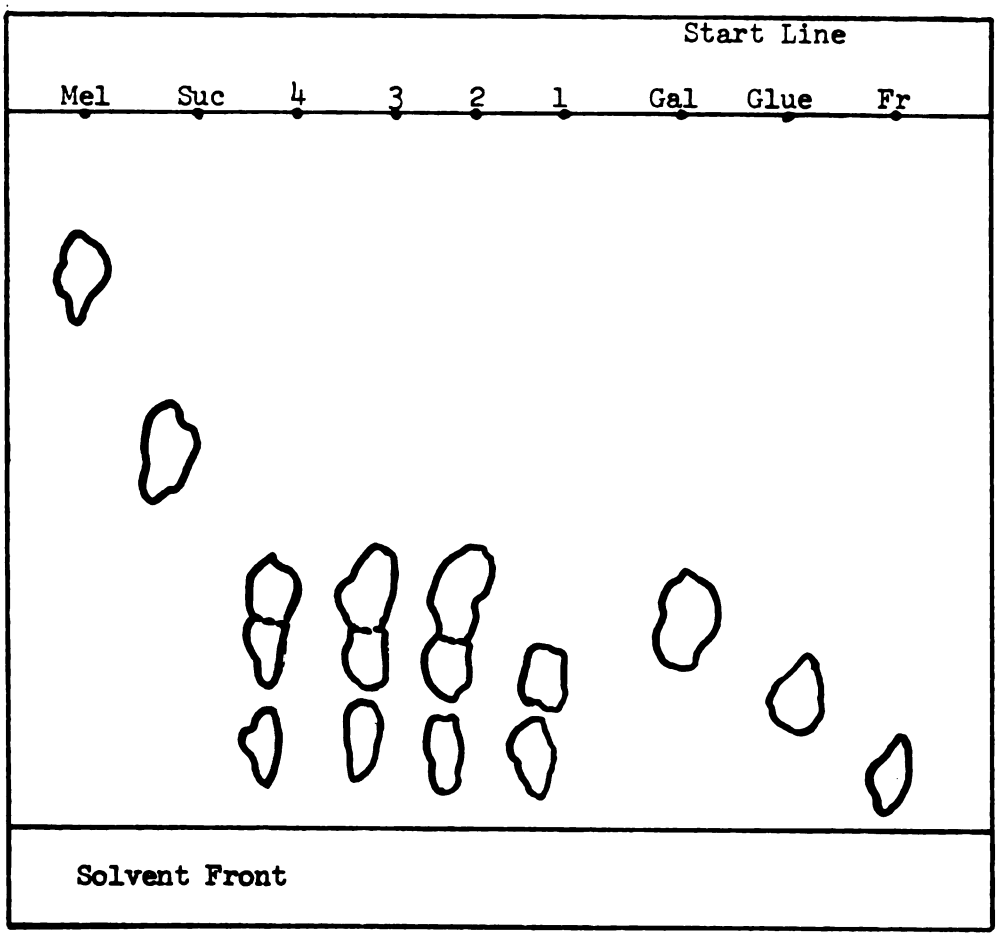


Figure 6. Descending paper chromatography of acid hydrolysates of cowpea oligosaccharides.

Variety: California No. 5 Cowpea.
Solvent: n-butanol, ethanol, water (5:3:2 by volume).
Fr., Fructose; Suc., Sucrose; Gluc., Glucose;
Gal., Galactose; Mel., Melibiose.
1 = Disaccharide; 2 = Trisaccharide; 3 = Tetrasaccharide;
4 = Pentasaccharide.

oligosaccharides (22, 23). In general, increasing the size of an oligosaccharide by one monosaccharide unit is found to decrease the logarithm of the partition function by an amount which depends on the type of hexose unit being added and its mode of attachment. Based on this principle, French and Wild in 1953 obtained linear relationships for sucrose, raffinose, stachyose, and verbascose, and for their hydrolysis products: D-glucose, melibiose, manninotriose, and verbascotetraose.

Similar relationships were obtained for the oligosaccharides separated from the cowpeas, Figures 7 and 8. This further supports the contention that the sugar occurring behind stachyose on the filter paper is a pentasaccharide and belongs to the same series of sugars as the confirmed ones: sucrose, raffinose, and stachyose; it is probably verbascose.

Oligosaccharide Content of the Cowpea Varieties

Table 4 shows the oligosaccharide content of the 13 cultivars analyzed. The sucrose, raffinose, and stachyose contents were determined by the phenol-sulfuric acid method (17). Recovery tests with this method gave sucrose 102.5%, raffinose 90.0%, and stachyose 97.5%. The resorcinol-HCl method (61) by which the verbascose content was determined was equally very reliable with 91.0% recovery.

Of the four oligosaccharides separated from the cowpeas, sucrose was the most variable in amount. It ranged from a low value of 1.8% to a high value of 3.1% with an average of 2.2% on a dry basis.

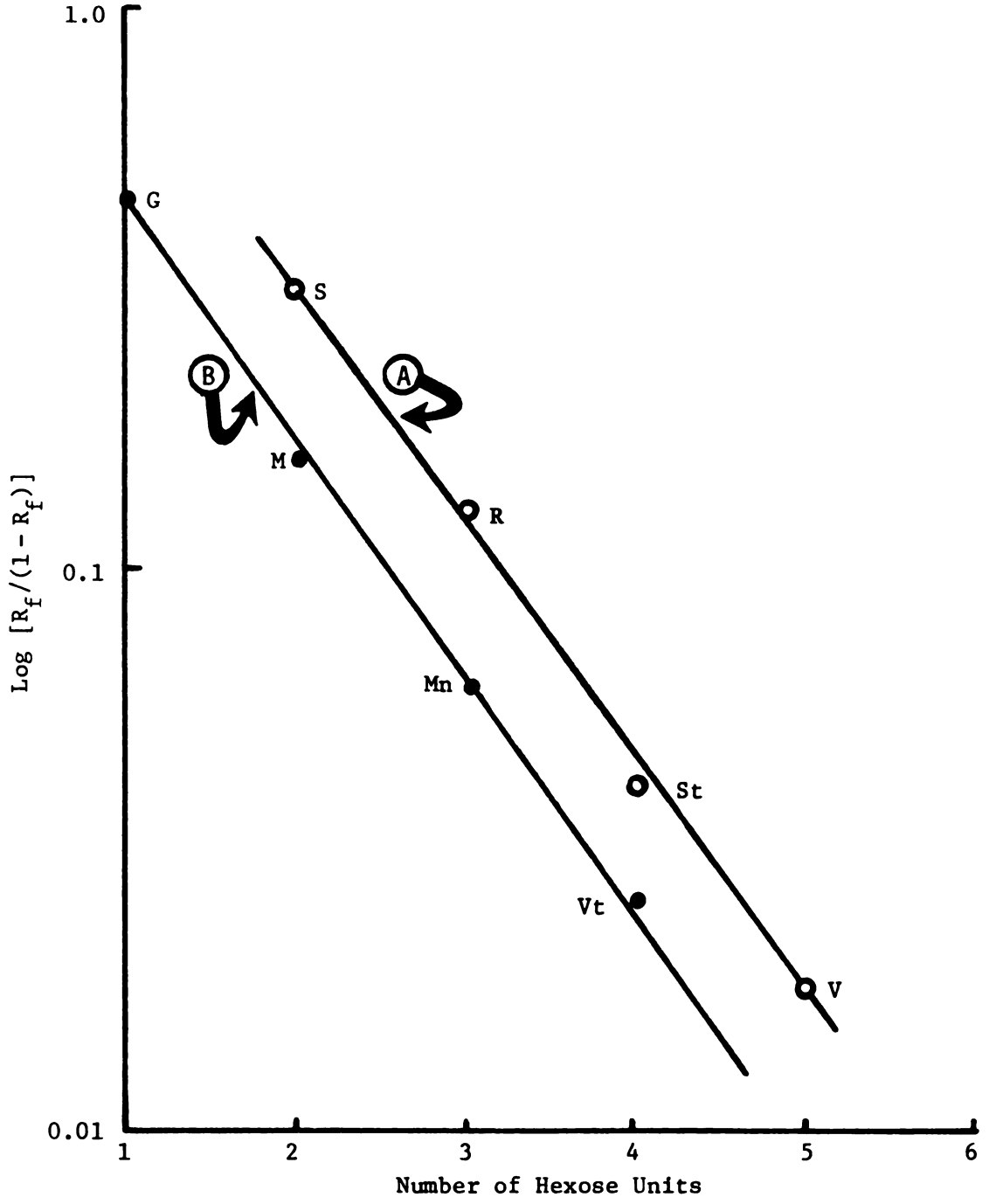


Figure 7. Plot of Log [$R_f/(1-R_f)$] against number of hexose units in cowpea oligosaccharides.

Solvent: n-butanol, ethanol, water, 5:3:2 by volume.
A: Sucrose terminal series, sucrose, raffinose, stachyose, and verbascose from cowpeas.
B: Glucose terminal series, glucose, melibiose, manninotriose, and verbascotetraose, obtained by invertase hydrolysis of sucrose, raffinose, stachyose and verbascose extracted from cowpeas.

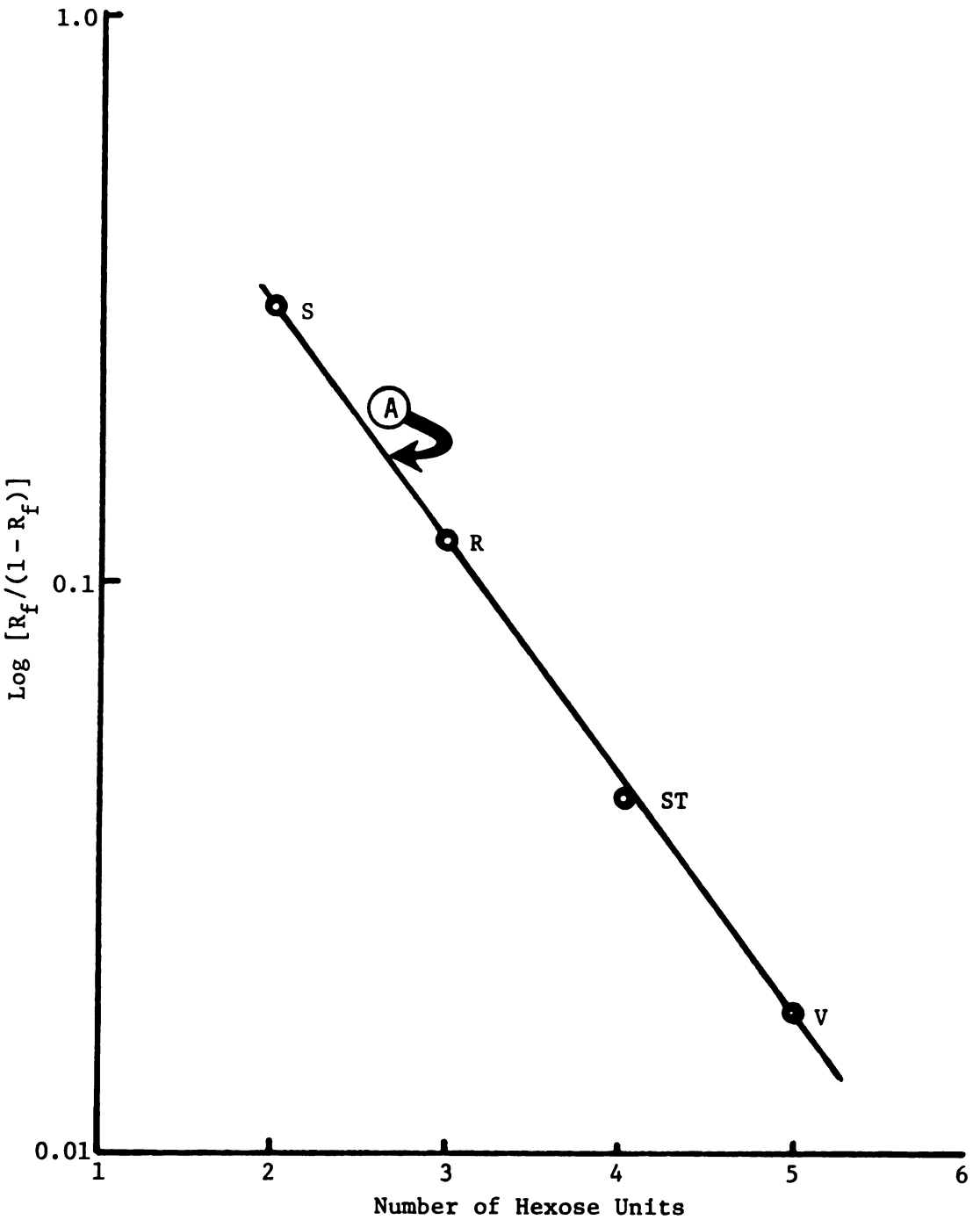


Figure 8. Plot of Log [$R_f/(1-R_f)$] against number of hexose units in cowpea oligosaccharides.

Solvent: 1-butanol, pyridine, water, 6:4:3 by volume.
A: Sucrose terminal series, sucrose, raffinose, stachyose, and verbascose from papergram separation of cowpea extract.

Table 4. Oligosaccharides of 13 Cowpea Cultivars (g/100 g dry basis)

Varieties	Sucrose	Raffinose	Stachyose	Verbascose
1 California No. 5	2.4[a]±0.09[b]	1.1 ± 0.08	3.6 ± 0.18	0.9 ± 0.05
2 Cabbage Cowpea	2.1 ± 0.04	1.2 ± 0.03	3.4 ± 0.11	0.9 ± 0.06
3 Aub. 70.3	1.8 ± 0.03	1.1 ± 0.05	3.1 ± 0.06	0.9 ± 0.01
4 Pinkeye Purple Hull	2.1 ± 0.01	1.3 ± 0.01	3.6 ± 0.03	0.9 ± 0.06
5 Mississippi Silver Skin	1.9 ± 0.05	1.1 ± 0.01	2.9 ± 0.09	1.0 ± 0.05
6 Magnolia Blackeye	2.4 ± 0.02	1.2 ± 0.06	3.4 ± 0.01	1.0 ± 0.01
7 Alabama 963.8 G1-5	1.8 ± 0.07	1.1 ± 0.05	4.1 ± 0.14	0.06±0.01
8 Alabama 562 3-1-2	1.9 ± 0.04	1.3 ± 0.04	3.5 ± 0.09	0.6 ± 0.03
9 Knuckle Purple Hull	1.8 ± 0.04	1.1 ± 0.08	3.4 ± 0.13	1.0 ± 0.03
10 Big Boy Cowpea	3.0 ± 0.06	1.1 ± 0.06	3.6 ± 0.01	1.0 ± 0.06
11 Sa Dandy Cowpea	2.0 ± 0.14	1.2 ± 0.02	3.3 ± 0.01	0.9 ± 0.06
12 Tenn. White-crowder	3.1 ± 0.05	1.1 ± 0.12	2.9 ± 0.21	0.8 ± 0.04
13 Giant Blackeye	2.6 ± 0.10	1.2 ± 0.19	3.8 ± 0.03	0.9 ± 0.02

[a]Mean of four determinations.

[b]One standard deviation.

Raffinose was fairly constant at about 1.2%. Stachyose was the most predominant sugar in almost all the varieties. The exception was in Tenn. Whitecrowder variety, where it fell slightly below sucrose. The mean value for stachyose was 3.4%. Two varieties, Mississippi Silver Skin and Tenn. Whitecrowder had the lowest stachyose value of 2.9% while the Alabama 963.8 G1-5 variety had the highest value of 4.1%. The rest were uniform at about 3.5%. Verbascose varied very little between varieties. The two Alabama varieties (Alabama 963.8 G1-5 and Alabama 562 3-1-2) were particularly low in verbascose with an average of 0.6% as compared to the others ranging from 0.8% to 1.0%.

The sucrose values compared fairly well with the values obtained by Nigam and Giri (49), and Lee et al. (43). The values for raffinose, stachyose, and verbascose did not agree with those obtained by either of these groups of workers. Nigam and Giri (49) reported the following values: sucrose 1.5%, raffinose 0.4%, stachyose 2.0%, and verbascose 3.1%. Lee et al. (43) obtained 1.86% for sucrose, 0.1% for raffinose, 1.66% for stachyose; they reported trace amount of verbascose.

The total oligosaccharide content of the cowpea varieties was fairly constant, Table 5. It ranged from 6.9% to 8.6% with an average of 7.7% on dry basis. Table 5 also shows the average and range of the non-digestible oligosaccharides (stachyose, raffinose, and verbascose) in the cowpeas. It shows that about 9% of the sugars of the cowpea are not digestible and hence contribute to the flatus problem that might be experienced by consuming the beans.

Table 5. Carbohydrates, Oligosaccharides, and Non-Digestible Oligosaccharides, Average and Range of the 13 Cultivars (g/100 g, dry basis)

	Average	Range
Total carbohydrates (excluding fiber)	63.6	61.3-65.7
Total oligosaccharides	7.7	6.9-8.6
Non-digestible oligosaccharides (stachyose, raffinose, verbascose)	5.5	4.8-5.9

CONCLUSIONS

The cowpeas contained a substantial amount of the raffinose group of oligosaccharides (raffinose, stachyose, and verbascose) together with sucrose. Sucrose varied considerably between varieties. Raffinose was fairly uniform, while stachyose and verbascose showed little variation.

Cowpea is also rich in protein and other important nutrients. In order, therefore, to utilize the full benefits of this crop, efforts should be made to evolve an efficient and a more economical way to eliminate the raffinose family of oligosaccharides from the beans and its by-products.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Albon, N., and Gross, D. 1950. The Chromatographic Determination of Raffinose in Raw Sugars, Analyst 75, 454.

2. Avigad, G., Amaral, D., Asensio, C., and Horecker, B. L. 1961. Galactodialdolase Production with an Enzyme from the Mold Polyporus Circinatus. Biochem. Biophys. Res. Commun. 4, 474-477.

3. Aykroyd, W. R., and Doughty, Joyce. 1969. Legumes in Human Nutrition (FAO Nutritional Studies No. 19). Food and Agriculture Organization of the United Nations. Rome.

4. Bailey, R. W. 1965. Oligosaccharides. Macmillan (Pergamon), New York.

5. Bailey, R. W., and Pridham, J. B. 1962a. The Separation and Identification of Oligosaccharides. Chromatog. Rev. 4, 114-136.

6. Bailey, R. W., and Pridham, J. B. 1962b. Oligosaccharides. Advan. Carbohydrate Chem. 17, 121-167.

7. Binkley, W. W., and Wolfrom, M. L. 1948. Chromatography of Sugars and Related Substances. Sugar Research Foundation, Inc., New York.

8. Bishop, C. T. 1964. Gas-Liquid Chromatography of Carbohydrate Derivatives. Advan. Carbohydrate Chem. 19, 95-147.

9. Block, R. J., Durrum, E. L., and Zweig, G. 1958. A Manual of Paper Chromatography and Paper Electrophoresis. 2nd ed. Academic Press, New York.

10. Boggs, L. A., Cuendet, L. S., Ehrenthal, I., Koch, R., and Smith, F. 1950. Separation and Identification of Sugars Using Paper Chromatography. Nature 166, 520.

11. Cassidy, H. G. 1957. Fundamental of Chromatography. Inter-Science, New York.

12. Cerning, J., Saposnik, A., and Guibot, A. 1975. Carbohydrate Composition of Horse Beans of Different Origins. Cereal Chem. 52(2), 125-138.

13. Cristofaro, E., Mottu, F., and Wuhrmann, J. J. 1972. Study of the Effect on Flatulence of Leguminous Seed Oligosaccharides. Cereal Sci. Today 17, 274.

14. Delente, J., and Landenburg, K. 1972. Quantitative Determination of the Oligosaccharides in Defatted Soybean Meal by Gas-Liquid Chromatography. J. Food Sci. 37, 372.

15. DeVerdier, C. H., and Hjelm, M. 1962. A Galactose-Oxidase Method for Determination of Galactose in Blood Plasma. Clin. Chem. Acta 7, 742-744.

16. Dobrick, L. A. 1958. Screening Method for Glucose and Blood Serum Utilizing Glucose Oxidase and an Indophenol Indicator. J. Biol. Chem. 231, 403-409.

17. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. 1956. Colorimetric Method for Determination of Sugars and Related Substances. Anal. Chem. 28, 350-356.

18. Foster, A. B. 1962. Zone Electrophoresis of Carbohydrates. Advan. Carbohydrate Chem. 12, 81-115.

19. Foster, A. B. 1962. Zone Electrophoresis (Ionophoresis). Methods Carbohydrate Chem. 1, 51-58.

20. Flood, A. E., Hist, E. L., and Jones, J. K. N. 1947. Quantitative Analysis of Sugars by the Method of Partition Chromatography. Part I. Standardization of Procedure. Nature 160, 86.

21. Floridi, A. 1974. Automatic Ion-Exchange Chromatography for Simultaneous Determination of Sugars and Sugar Alcohols. Science e Technologia degli Alimenti 4(11), 39-42.

22. French, D. 1954. The Raffinose Family of Oligosaccharides. Advan. Carbohydrage Chem. 9, 149-184.

23. French, D., and Wild, G. M. 1953. Correlation of Carbohydrate Structure with Papergram Mobility. J. Am. Chem. Soc. 75, 2612.

24. Gomoyo, T., and Nakamura, N. 1966. Biosynthesis of Raffinose From Uridine Diphosphate Galactose and Sucrose by an Enzyme Preparation of Immature Soybeans. Agr. Biol. Chem. 30, 425-427.

25. Gould, M. F., and Greenshields, R. N. 1964. Distribution and Changes in the Galactose--Containing Oligosaccharides in Ripening and Germinating Bean Seeds. Nature 202, 180-109.

26. Hardinge, M. G., Swarner, J. B., and Crooks, H. 1965. Carbohydrates in Foods. J. Am. Dietet. Assoc. 46, 197-204.

27. Havel, E., Tweeten, T. N., Seib, P. A., Wetzel, D. L., Liang, Y. T., and Smith, O. B. 1977. Oligosaccharides Released During Hydration of Textured Soy as Determined by High Performance Liquid Chromatography. J. Food Sci. 42, 666-668.

28. Hawthorne, J. R. 1947. Mico-Estimation of Sugars Separated on the Filter Paper Chromatogram. Nature 160, 714.

29. Heftmann, E. 1967. Chromatography. 2nd ed. Reinhold Publishing Corp., New York.

30. Horrocks, R. H. 1949. Paper Partition Chromatography of Reducing Sugars with Benzidine as a Spraying Reagent. Nature 164, 444.

31. Hough, L., and Jones, J. K. N. 1962a. Chromatography on Paper. Methods Carbohydrate Chem. 1, 21-31.

32. Hough, L., and Jones, J. K. N. 1962b. Enzymic Methods for Determination of D-Glucose. Methods Carbohydrate Chem. 1, 400-419.

33. Hugget, A. St. G., and Nixon, D. A. 1957. Enzymic Determination of Blood Glucose. Biochem. J. 66, 120.

34. Jeanes, A., Wise, C. S., and Dimler, R. J. 1951. Improved Techniques in Paper Chromatography of Carbohydrates. Anal. Chem. 23, 415-420.

35. Joslyn, M. A. 1970. Methods in Food Analysis. 2nd ed. Academic Press, Inc., New York.

36. Jones, J. K. N., and Pridham, J. B. 1953. A Colorimetric Estimation of Sugars Using Benzidine. Nature 172, 161.

37. Kawamura, S. 1967. Quantitative Paper Chromatography of Sugars of the Cotyledon, Hull, and Hypocotyl of Soybeans of Selected Varieties. Kagowa Diagakw Nogokubu Gakujustu Hokuku 18, 117-131.

38. Kawamura, S. 1968. Changes of Sugar and Decrease in Available Lysine on Autoclaving Defatted Soybean Flakes. J. Jap. Soc. Food Nutr. 20, 478-481.

39. Keilin, D., and Hartree, E. F. 1948. The Use of Glucose Oxidase (Notatin) for the Determination of Glucose in Biological Material and for the Study of Glucose-Producing Systems by Manometric Methods. Biochem. J. 42, 230-237.

40. Kim, W. J., Smith, C. J. B., Nakayama, T. O. M. 1973. Removal of Oligosaccharides from Soybeans. Lebensmittel-Wissenschaft + Technologie 6(6), 201-204.

41. Kircher, H. W. 1962. Gas-Liquid Partition Chromatography of Sugar Derivatives. Methods Carbohydrate Chem. 1, 13-20.

42. Korytnyk, W., and Metzler, E. 1962. Formation of Raffinose and Stachyose in Lima Beans. Nature 195, 616-617.

43. Lee, C. Y., Shallenberger, R. S., and Vittum, M. T. 1970. Free Sugars in Fruits and Vegetables. New York's Food and Life Sciences. Bull. 1. Food Science, Cornell University. Geneva, New York.

44. Lemieux, R. V., and Bauer, H. F. 1954. Spray Reagents for the Detection of Carbohydrates. Anal. Chem. 26, 920.

45. Lineback, D. R., and Ke, C. H. 1975. Starches and Low Molecular Weight Carbohydrates for Chickpea and Horse Bean Flours. Cereal Chem. 52, 334.

46. Lunt, E., and Sutcliffe, D. 1953. A New Colorimetric Reagent for Carbohydrates. Biochem. J. 55, 122.

47. Mattson, A. M., and Jensen, C. O. 1950. Colorimetric Determination of Reducing Sugars with Triphenyltetrazolium Chloride. Anal. Chem. 22, 182.

48. Myers, V. C., and Croll, H. M. 1921. The Determination of Carbohydrates in Vegetables. J. Biol. Chem. 46, 537.

49. Nigam, V. N., and Giri, K. N. 1961. Sugars in Pulses. Canadian J. Biochem. Physiol. 39, 1847.

50. Official Methods of Analysis of the Association of Official Analytical Chemists 1970. 11th ed. Published by the Association of Official Analytical Chemists. Washington, D.C.

51. Oyenuga, V. A. 1968. Nigeria's Foods and Feeding Stuffs. Ibadan University Press.

52. Ovodov, Yn. S., Evtushenko, E. V., Vaskovsky, V. E., Ovodova, R. G., and Soloveva, T. F. 1967. Thin-Layer Chromatography of Carbohydrates. J. Chrom. 26, 111.

53. Palmer, J. K. 1975. A Versatile System for Sugar Analysis Via Liquid Chromatography. Anal. Letters 8(3), 215-224.

54. Pastuska, G. 1961. Untersuchungen Uber Die Quantitative und Quantitative Bestimmung der Zucker Mit Hilfe der Kieselgelschict-Chromatographie. Z. Anal. Chem. 179, 427.

55. Patridge, S. M. 1946. Application of Paper Partition Chromatography to the Quantitative Analysis of Reducing Sugars. Nature 158, 270.

56. Patridge, S. M., and Westhall, R. G. 1948. Filter-Paper Chromatography of Sugars. Biochem. J. 42, 238-248.

57. Patridge, S. M. 1949. Aniline Hydrogen Phthalate as a Spraying Reagent for Chromatography of Sugars. Nature 164, 443.

58. Pritchard, P. J., Dryburg, E. A., and Wilson, B. J. 1973. Carbohydrates of Spring and Winter Field Beans. J. Sci. Food Agric. 24, 663.

59. Rackis, J. J. 1974. Oligosaccharides of Legumes: Alpha-Galactosidase and the Flatus Problem. Abstract of Papers, American Chem. Soc. 168, CABB 48.

60. Raybin, H. 1937. Direct Demonstration of the Sucrose Linkage in the Oligosaccharides. J. Am. Chem. Soc. 59, 1402.

61. Roe, J. H. 1934. A Colorimetric Method for the Determination of Fructose in Blood and Urine. J. Biol. Chem. 107, 15.

62. Roth, H., Segal, S., and Bertoli, D. 1965. The Quantitative Determination of Galactose--An Enzymic Method Using Galactose, with Applications to Blood and Other Biological Fluids. Anal. Biochem. 10, 32-52.

63. Schultz, H. W., Cain, R. F., and Wrolstad, R. W. 1969. Symposium on Foods. Carbohydrates and Their Roles. Avi. Publ. Coy Inc., West Port, Conn.

64. Shallenberger, R. S., and Moores, R. G. 1957. Quantitative Determination of Reducing Sugars and Sucrose Separated by Paper Chromatography. Anal. Chem. 29, 27.

65. Sipple, H. L., and McNutt, K. W. 1974. Sugars in Nutrition. Academic Press, New York.

66. Smartt, J. 1976. Tropical Pulses. Tropical Agric. Services. Longman Group Ltd., London.

67. Smith, I. 1958. Chromatographic Techniques: Clinical and Biochemical Applications. Heineman Medical Books, Ltd., London, Interscience, New York.

68. Snauwaert, F., and Markakis, P. 1976. Effect on Germination and Gamma Irradiation on the Oligosaccharides of Navy Beans. Lebensm-Wiss, U-Technol. 9, 93-95.

69. Stahl, E. 1961. Thin-Layer Chromatography. Z. Anal. Chem. 181, 303.

70. Stahl, E., and Kaltenbach, U. 1961. Dunnschict-Chromatographic. VI. Mitteilung Spurenanalyse von Zuckergemischen Auf Kieselgur G-Schichten. J. Chroma. 5, 351.

71. Steegerda, F. R. 1961. Relation Between Consumption of Dry Beans and Flatulence in Human Subjects. Dry Beans Res. Conf. 14-15 (edited by Western Regional Research Laboratory, Albany, California).

72. Tanaka, M., Thananunkul, D., Lee, T. C., and Chichester, C. O. 1975. A Simplified Method for the Quantitative Determination of Sucrose, Raffinose and Stachyose in Legume Seeds. J. Food Sci. 40, 1087.

73. Tanner, W., and Kandler, O. 1966. Biosynthesis of Stachyose in Phaseolus Vulgaris. Plant Physiology 41, 1540-1542.

74. Trevelyan, W. E., Procter, D. P., and Harrison, J. S. 1950. Detection of Sugars on Paper Chromatograms. Nature 166, 444.

75. Whistler, R. L., and Bemiller, J. N. 1962. Quantitative Paper Chromatography: Quantitative Paper Chromatography Micro-Determination of Carbohydrates. Methods in Carbohydrate Chem. 1, 395-399.

76. Whistler, R. L., Hough, L., and Hylin, J. W. 1953. Determination of D-Glucose in Corn Syrups. Anal. Chem. 25, 1215-1216.

77. Whitehouse, K., Zarow, A., and Shay, H. 1945. Rapid Method for Determining "Crude Fiber" in Distiller's Dried Grain. J. Assoc. Offic. Agric. Chem. 28, 147-152.

78. Williams, K. T., and Bevenue, A. 1951. A Simple Technique for the Identification of Raffinose and Sucrose by Enzymic Hydrolysis on Paper Chromatograms and the Subsequent Separation of the Hydrolyzed Products by Paper Chromatography. Science 113, 582.

79. Williams, K. T., Potter, E. F., Maher, W. B., Wison, J. R., and Wright, H. M. 1960. Sugars in Plants. Methods for Fructose. J. Assoc. Offic. Agric. Chemists 43, 512-514.

80. Yau-Lai-Lo, W., Steinkraus, K. H., Hand, D. B., Hackler, L. R., and Wilkens, W. F. 1968. Soaking Soybeans Before Extraction as It Affects Chemical Composition and Yield of Soy-Milk. Food Technol. (Chicago) 22, 138-140.