CARBOHYDRATES OF THE COFFEE BEAN

DISSertation
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by

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I. INTRODUCTION AND STATEMENT OF PROBLEM

The estimated 1955-1956 world exportable production of coffee is two and one-half million tons. The value of United States yearly coffee imports (60-70% of the world crop) is nearing three billion dollars.

Despite its tremendous economic importance and its widespread use, comparatively little fundamental chemical knowledge of coffee is available. The roasting process is little understood although it is well known that the flavor of coffee cannot be duplicated by the roasting of any other seed. The recent introduction of soluble coffee extracts on the market has stimulated fundamental research on coffee.

Since carbohydrates constitute 50-60% of the coffee bean, it would be expected that these substances would play a significant role in the roasting process. Thus, it was felt that a fundamental study of these carbohydrates might contribute significantly to man's knowledge of coffee. This work is the beginning of such a study.
II. HISTORICAL REVIEW

A. Carbohydrates of the Coffee Bean

1. Sugars

There can be little doubt concerning the presence of sucrose in green coffee beans. This is not unexpected in view of its widespread occurrence in plant materials. Graham, Stenhouse, and Campbell (1) reported the crystallization of cane sugar from an infusion of raw coffee. Many others (2-8) also found sucrose in varying amounts. Probably the most reliable data are those of Ewell (2) and of Slotta and Neisser (8).


Ewell (2) extracted powdered (1 mm.) green coffee beans first with absolute ether and then with 70% ethanol. The alcohol was removed by evaporation and the remaining aqueous solution was treated with basic lead acetate. Excess lead was removed with hydrogen sulfide and the sucrose was hydrolyzed with acid. Sucrose was estimated in an aliquot part of the solution, the cuprous oxide being determined electrolytically. Duplicate determinations gave 6.24% and 6.34% sucrose. These numbers included a small amount of reducing sugar that was also present.

Slotta and Neisser (8) exhaustively extracted green coffee, defatted with petroleum ether, with water and after purifying the extract with lead acetate as usual, determined the amount of sucrose from the change in rotation and from the change in reducing power. Calculated from the change in rotation, the green coffee bean contained 6.25% sucrose, while from the change in reducing power the value was 6.50%.

Contrary to the above results, Levesie (9), and Herfeldt and

(9) O. Levesie, Arch. Pharm., 8, 294 (1876).

Stutzer (10) could find no sucrose in green coffee beans, while


Bell (11) reported a peculiar sugar, which was not hydrolyzed by

boiling with dilute acetic acid, and which yielded only glucose when treated with dilute hydrochloric acid. Graf, however, found the sugar of coffee beans hydrolysable by dilute acetic acid. It must be concluded that sucrose is present in the green coffee bean to the extent of somewhat more than 6%.

There is much disagreement in the literature concerning the occurrence of free reducing sugars in the green coffee bean. Payen (12) first reported glucose in green coffee beans. He found

(12) A. Payen, Compt. rend., 23, 244 (1846).

glucose (probably sucrose), starch, dextrin, and vegetable acids to comprise 15.5% of the bean. The next reference to reducing sugar is by Ewell (2) although he cautions that it may have resulted from partial hydrolysis of the sucrose present. Warnier (13) found 0.5%


and Commaille (14) found 2.6% glucose (reducing power calc. as


glucose?) in green coffee beans. Much earlier, Hlasiwetz (15) claimed


to have obtained glucose by hydrolysis of caffestannic acid. Slotta and Neisser (8) found 1.0% reducing sugar but were unable to detect glucose explicitly.
Balanced against these results are the findings of Herfeldt and Stutzer (1) and Graf (4) who could detect no reducing sugars in the free state. Herfeldt reported glucose present as a glycoside.

Schulze (3) reported an unidentified sugar in coffee beans which was difficultly soluble in 95% alcohol.

2. Polysaccharides

Payen (12) was the first investigator to determine the amount of polysaccharide material in the coffee bean. He reported that glucose (probably sucrose), dextrin and vegetable acids comprised 15.5% and cellulose 3.4% of the bean. No further work was done on the sugar polymers until Levesie (9) determined the amount of "mucilage" present.

Levesie (9) macerated ground green coffee beans with water and then added an amount of alcohol which produced a 67% alcohol solution. The extraction was continued for 24 hours and then filtered. The residue was hydrolyzed with ca. 4% sulfuric acid, filtered, and reducing sugars determined in the filtrate. Levesie, assuming starch was absent, calculated the total reducing sugars as mucilage (21-28%). The term "mucilage" as used by Levesie corresponds to the more recent designation "easily hydrolyzable polysaccharides" or roughly to "hemicellulose".

Reiss (16) discovered what he considered to be a new type of

(16) R. Reiss, Ber., 22, 609 (1889).

sugar among the products of mild hydrolysis of the water-insoluble matter. He described its properties and derivatives and designated
it, "seminose". Schulte (3) later identified "seminose" with mannose.

Shortly after the work of Reiss, Schulze, Steiger, and Maxwell (17)


investigated the green beans of *Coffea arabica*. Among the products of hydrolysis with 5% $\text{H}_2\text{SO}_4$ of the ether-, hot alcohol-, and 0.2% sodium hydroxide-extracted residue, they found mannose and galactose. An amount of mucic acid equivalent to ca. 50% galactose was obtained by oxidation with nitric acid. The residue from the mild hydrolysis yielded glucose and a considerable quantity of mannose on further hydrolysis with 75% sulfuric acid. After boiling the water-insoluble material with phloroglucinol and hydrochloric acid only a weak red color was obtained. Only a little furfural was obtained by the method of Stone and Tollens (18) (distillation with hydrochloric acid and precipitation of the furfural with ammonia).


According to Whistler and Smart (19), the paper by Schulze,


Steiger, and Maxwell (17) is the first record of a galactomannan.

Ewell (2) also was concerned with the polysaccharides. He extracted the finely ground beans with absolute ether and then with 75% alcohol in a Soxhlet extractor. The water-insoluble portion
yielded an abundance of furfuraldehyde when distilled with hydrochloric acid. Distillation of 5 grams of untreated coffee gave furfural equivalent to 8-10% pentosan.

A gummy precipitate was obtained when a 5% sodium hydroxide extract was diluted with alcohol according to the method used for the separation of xylan from wood. After purifying by washing with alcoholic hydrochloric acid, alcohol, and ether and drying over sulfuric acid, a slightly greyish, translucent, hard, and brittle mass was obtained. The substance, easily reduced to a powder in a mortar, was hydrolyzed with 5% sulfuric acid, yielded reducing sugars equivalent to 74.2% glucose. By distillation with hydrochloric acid it gave 13% furfuraldehyde, by oxidation with nitric acid, 18.7% of mucic acid, and on ignition left 0.84% ash. Ewell suggested a compound containing equal numbers of galactose and pentose units. No considerable amount of mannose was found.

Schulze (3) soon repeated the investigation of coffee beans and found pentosans, confirming Ewell's work. The presence of large amounts of mannose was also confirmed. Schulze treated the residue from the hot alcohol extraction with cold dilute ammonium hydroxide. The brownish green extract was decanted and the residue was washed thoroughly with water and dried. This residue yielded furfural, isolated as the phenylhydrazone according to the method of Chalmot and Tollens (20), equivalent to 6.72% pentosan on a dry-weight basis.

(20) O. de Chalmot and B. Tollens, Ber., 24, 694 (1891).
This same residue, when boiled with 1.25% sulfuric acid, yielded a hydrolyzate containing sugars. Neutralized, concentrated to a sirup, and oxidized with nitric acid, it yielded a considerable amount of mucic acid.

The residue that was insoluble in 1.25% sulfuric acid was treated with hydrochloric acid and potassium chlorate and then with hot dilute ammonium hydroxide, Hoffmeister cellulose determination (21).


The material still insoluble was hydrolyzed with 75% sulfuric acid yielding a sugar sirup which was rich in mannose. In another publication (22) Schulze reported that the mannose phenylhydrazone (m.p. 185-186°) was levorotatory in hydrochloric acid solution indicating that the sugar was D-mannose. Glucose was identified as the osazone, m.p. 202-203°, after the removal of mannose. Assuming the glucose was the D form, it was calculated from the specific rotation of the hydrolyzate, $[\alpha]_D + 27.1°$, that the residue was ca. 60% mannose and 40% glucose. No other sugars were detected in the hydrolyzate.

The mannose-yielding carbohydrate was found by Schulze to resist hydrolysis by hot dilute hydrochloric acid, F. Schulze's reagent (15 parts of nitric acid, sp. g. 1.15, to 1 part potassium chlorate by weight) and Hoffmeister's cellulose-determination mixture of hydrochloric acid and potassium chlorate. It was soluble in ammoniacal
copper solution and in a mixture of hydrochloric acid and zinc chloride. Since these characteristics were like cellulose, he called it manno-cellulose.

It is very probable that most of the polysaccharide preparations of the early workers were far from uniform in regard to the type of sugar polymer contained in them. That Schulze's manno-cellulose was not homogeneous was soon demonstrated by E. Gilson (23). Gilson succeeded in isolating the mannose-yielding carbohydrate from the cellulose by dissolving the mixture in ammoniacal copper solution and precipitating cellulose with carbon dioxide. When the solution was concentrated and the residue treated with hydrochloric acid the mannan was recovered in a pure state. According to Gilson, the mannan differed from cellulose only in that it did not give a blue color with zinc iodochloride.

Surprisingly, Baker (24) detected no galactose in the coffee bean. He reported that a principal constituent of the coffee berry was a manno-arabinose or manno-xylose which yielded 80-90% mannose on hydrolysis. No details of his work were published.

K. Gorter (25) described a pectic substance which was precipitated


from a 60% ethanol percolate of green coffee beans by the addition of an equal amount of 96% alcohol. This substance was precipitated by ammoniacal copper solution, was dextrorotary \([\alpha]_D^25 = +15^\circ\), and gave no color with iodine or iodine and sulfuric acid. It was hydrolyzed by warming with dilute mineral acid. Oxidation of the pectin with nitric acid gave mucic acid equivalent to 15% galactose in the dry substance. When warmed with phloroglucinol and hydrochloric acid a red color developed, indicating a pentose. (It should be noted that galacturonic acid is a common constituent of pectic substances and also gives a red color when treated with phloroglucinol and hydrochloric acid.)

Savur and Sreenivasan (26) found no pectic material in a sample of coffee "seed".

Täufel and Thaler (27) undertook the study of the cell wall components of the coffee bean. The beans were dried for three days over phosphorus pentoxide, then ground and extracted for three days with ether. The ether-free residue was twice extracted with water by stirring and was then dried.

The resulting insoluble residue (52.6% of the dry weight of the original bean) was considered to consist of "albumin, resins, minerals
of the cell wall, lignin, a galactose part, and a glucose part. This material was ground dust-fine and dispersed in a 0.25% chlorine dioxide solution. A small amount of pyridine was added and the reaction was allowed to go to completion at room temperature (6 days).

The insoluble residue (the "glycose part", 47.0% of the dry weight of the original bean) was filtered and dried over phosphorus pentoxide, yielding a white powder. This glycose part was treated with 5% sodium hydroxide for 24 hours to dissolve mannans and xylans. The solution was filtered and acetic acid was added to the filtrate. The flocculent precipitate which formed was extracted for three days in a Soxhlet apparatus with methanol to remove sodium acetate and acetic acid and then with ether to remove methanol. The residue from the sodium hydroxide extraction was free of pentosans.

This residue was placed in 75% sulfuric acid for 24 hours, then diluted to 7.5% sulfuric acid and the hydrolysis completed by heating under reflux for 2.5 hours. A sirupy hydrolysis product resulted which was identified as mannose by its phenylhydrazone.

Another portion of the glycose part was extracted with 5% sodium hydroxide. The mannan- and xylan-containing solution was diluted with twice its volume of 96% ethanol. The precipitate containing both carbohydrates was extracted with methanol and ether as before. The substance contained 14.7% xylan, xylose being identified as the phenylhydrazone.

The residue left from the sodium hydroxide extraction was again treated with 5% sodium hydroxide and then tested for cellulose. The
material was subjected to acetolysis. A crystalline product melting at 220°C, octaacetylcellobiose, proved the presence of cellulose.

Quantitative determinations showed 15.3% mannan, 1.14% xylan, (2.14% furfural-yielding material, some of which was extracted by water), and 29.9% cellulose, calculated by difference in the dry bean.

Slotta and Neisser (6) investigated the green coffee bean with the objective of achieving a 100% component total analysis. Most of their values were obtained according to the methods of Waksman and Stevens (28).


The dried beans were successively extracted with petroleum ether, hot water, and hot 95% alcohol. The residue from the alcohol extraction was refluxed for 5 hours with 2% hydrochloric acid. The reducing sugars liberated, determined by the method of Bertrand (29), corresponded to 20.2% hemicelluloses.


The residue from the dilute hydrochloric acid extraction was hydrolyzed with 80% sulfuric acid at room temperature for 2.5 hours, then with 5% sulfuric acid under reflux for 5 hours. The reducing sugars, determined by the method of Bertrand, corresponded to 11.2% cellulose.
3. Carbohydrases

Helferich and Vorsatz (30) investigated coffee bean emulsin.


They found the most active enzymes to be $\alpha$-$D$-mannopyranosidase, $\alpha$-$D$-galactopyranosidase, and $\beta$-$D$-galactopyranosidase. Coffee emulsin is similar to lucerne emulsin in respect to its $\alpha$-$D$-mannopyranosidase and $\alpha$- and $\beta$-$D$-galactopyranosidase activity. It differs in that its $\beta$-$D$-glucopyranosidase activity is small and only proved, and in some degree measured, with special substrates.

Toward heating in water and toward change in pH, coffee emulsin is similar to lucerne emulsin. The $\alpha$-$D$-mannopyranosidase is somewhat resistant to heating.

Coffee emulsin and lucerne emulsin are similar in several respects (see above) but both differ from sweet almond emulsin and from each other in characteristic ways.

Sweet almond emulsin splits $o$-cresyl $\beta$-$D$-glucopyranoside and galactopyranoside remarkably faster than the corresponding phenyl compound. The same is true for the $\beta$-$D$-glucopyranosidase of lucerne emulsin but not for the $\beta$-$D$-galactopyranosidase of lucerne emulsin. Coffee emulsin acts similarly. It has been found that sweet almond emulsin splits derivatives of vanillin, protocatechualdehyde, and caffeic acid still faster.

As with lucerne emulsin, coffee emulsin splits phenyl $\beta$-$D$-galactopyranoside (value = 0.0029) and 4-formyl-2-methoxyphenyl
β-D-galactopyranoside (vanillin β-D-galactopyranoside) (value = 0.0027) with practically identical speeds. However, the β-D-glucopyranosides of vanillin, protocatechualdehyde, and caffeic acid were split so much faster by coffee emulsin than were phenyl β-D-glucopyranoside or salicin, that they are suggested for a measure of the β-D-glucopyranosidase activity of coffee emulsin since the usual salicin test is not certain.

A weak diastase activity was noted but not further investigated.

B. Current Techniques Used in the Investigation of Plant Materials

1. Plant Extracts

One of the prime considerations in preparing a plant extract is the avoidance of enzyme action on the constituents being isolated. Plant enzymes can rapidly alter the carbohydrate content of plant materials. Wylam (31) made a study of the changes in D-glucose,


D-fructose, sucrose, fructan, and dry matter which take place in grass samples after cutting. The observed increase in dry matter from 18.4% in the freshly cut grass to 44.6% in grass wilted 24 hr. in air is particularly striking. Changes in the amounts of the simpler sugars were also noted.

Reifer and Melville (32) claimed to reduce enzyme action to a

minimum by ice-cold water extraction for ca. 10 min. in a blender.
The final temperature of the macerate was about 8°.

The most commonly used method of tissue extraction aims at inactivation of the enzymes with ethanol (33). As soon as possible after collection and maceration the tissues are placed in boiling ethanol for a short time. Following this, water is added to produce a final concentration of 80% ethanol, taking into account the moisture content of the material. It is also advisable to add a small amount of calcium carbonate to prevent hydrolysis of labile compounds by any organic acids present. The mixture is then placed in sealed containers and allowed to stand at least 24 hr. to permit thorough penetration of the solvent into the tissues. Finally the mixture is filtered and the residue washed several times with 80% ethanol. If a quantitative extraction is desired, the material can be further extracted in a Soxhlet apparatus with 80% ethanol.

The upper limit of the oligosaccharides extracted by 80% ethanol has not been exactly defined. However, Burrell (33) suggests that it will not remove appreciable amounts of penta- and higher oligosaccharides. In addition this method has the advantage that it does not remove starch.

Clarification of plant extracts is often carried out, particularly before the quantitative determination of sugars. Williams,
Bevenue, and Washauer (34) studied the effects of the lead acetate

(34) K. T. Williams, A. Bevenue, and B. Washauer, J. Assoc.

and charcoal methods of clarification on the reducing sugars in 29
different plant tissues. In about half of the extracts no clarifi-
cation was necessary. In the remainder of the extracts the charcoal
treatment satisfactorily clarified the extract for the sugar
determination.

The use of ion-exchange resins in the clarification of plant
extracts has been examined by Williams, Potter, Bevenue, and
Scurzi (35). They found ion-exchange resins to be a superior means

(35) K. T. Williams, E. F. Potter, A. Bevenue, and W. R.

of removing interfering ionic substances from plant extracts. Control
experiments with pure D-glucose, D-fructose, and sucrose showed no
change in the concentrations of these sugars.

Other workers (36, 37, 38) have noted deleterious effects when

(36) S. Roseman, R. H. Abeles, and A. Dorfman, J. Biol. Chem.,
36, 232 (1952).


(38) L. Rebenfeld and E. Pacsu, J. Am. Chem. Soc., 75, 4371
(1953).

treating solutions of reducing sugars with strongly basic resins.

The investigation of the sugars, amino acids, and other
constituents of plant extracts has been immensely simplified in recent years by the advent of the various chromatographic methods (39-45). The applications of many of these methods to plant analysis (39) A. J. P. Martin and R. L. M. Synge, Biochem. J., 35, 1358 (1941).


(42) S. M. Partridge, J. Biol. Chem., 12, 238 (1948).


(45) A. B. Foster, Chemistry and Industry, 1050 (1952).

are well covered in Modern Methods of Plant Analysis (46).


2. Plant Polysaccharides

The modern methods of polysaccharide chemistry have been critically summarized in a recent monograph by Whistler and Smart (47).


Much of the material of this section has been drawn from their book.

a. General Procedures

The removal of a polysaccharide from its natural source and its
Isolation in a pure form suitable for chemical investigation is a formidable task. As it occurs in nature, a polysaccharide is usually non-uniform in length; it is accompanied by saccharases which can degrade it during isolation and by other polysaccharides of similar solubility characteristics. Beyond this, polysaccharides of different structure are often held tightly together by secondary valence bonds (and in some instances primary valence bonds) and by mechanical entanglement. Inasmuch as solution and fractional precipitation are the chief means of separating polysaccharides, the difficulties encountered in the elucidation of the nature of glycans are readily apparent.

It is generally advisable when isolating polysaccharides to inactivate the accompanying enzymes by immersing the material in ethanol, often with heating. Some enzymes, notably β-D-glucopyranosidase, are resistant to inactivation by both ethanol and heat. Most enzymes are denatured by the combination of ethanol and temperatures above 70°. Enzymes may also be inactivated by salts of heavy metals.

Drying of plant tissues presents another problem since a pronounced decrease in the solubility of the polysaccharides may occur (probably due to the chains coming into contact and forming secondary bonds). Fortunately, insolubilization can be obviated by special techniques of drying. Optimum drying requires rapid removal of water at low temperatures (ca. 0-40°). This may be accomplished by vacuum drying in an oven, by lyophilization, or by thorough washing of the finely divided material with successive portions of
alcohol and desiccation over calcium chloride.

The next step is often the removal of fats and waxes which would hinder the penetration of hydrophilic solvents. The fats may be extracted with aqueous methanol, ethanol, or the benzene-ethanol azeotrope. Hydrophilic solvents are better able to penetrate the hydrophilic material.

Impurities are usually extracted from cellulose but most other polysaccharides are isolated by selective extraction of the desired species or fractional precipitation from solution. The purity of a polysaccharide is difficult to determine. Freedom from other polysaccharides is indicated when the polysaccharide can be separated by at least two different procedures into fractions, each of which has the same analysis and properties as other fractions in the group. A polysaccharide is always heterogeneous with respect to chain length. The range of molecular size will depend on the uniformity with which they are produced naturally and the amount of degradation they undergo during isolation.

b. Holocellulose Preparation

The method of isolation of any plant polysaccharide will have certain unique aspects arising from the nature of the starting material and accompanying impurities. However, experience has led to a general procedure for the systematic isolation of plant polysaccharides, particularly the cell-wall polysaccharides. This procedure is preparation of the holocellulose and is specifically intended to remove lignin which interferes with the extractive removal of hemicelluloses. Depending upon the method, chlorine or
chlorine dioxide is the active agent. Usually all readily soluble substances (lipids, sugars, etc.) are removed prior to the chemical treatment.

When any appreciable amount of lipids are present it is usually advisable to remove them as this opens the tissues to penetration by hydrophilic solvents. Lipids themselves are not completely removed by hydrophobic solvents. It has been found that 2:1 benzene/ethanol (by weight, approx. the azeotrope) is an excellent lipid extraction solvent. The mixture of hydrophobic and hydrophilic solvents provides complete penetration of the tissue.

Depending upon the amount of water-soluble substances in the tissues, one or more aqueous extractions may be desirable. Hot water will ordinarily extract more material than cold water but the increased danger of hydrolysis and the possibility of removing low molecular weight fractions of so-called water-insoluble polysaccharides should be noted.

Pectic substances are usually removed by extracting for one or more separate periods with 0.5% solutions of ammonium oxalate at 90-100°.

Erich Schmidt and co-workers (48) developed the first method for


the oxidative removal of lignin from plant tissues. The treatment involved long standing (up to three weeks) in water containing
pyridine and chlorine dioxide. This method was used by Tüfelf and Thaler (27) in their investigation of the cell-wall polysaccharides of the coffee bean.

A short time later Kurth and Ritter (49) devised a method which


in its present form requires successive treatment of the ground tissue at 75° with water, chlorine, ethanol, and a 3% solution of ethanolamine in ethanol until the residue remains uncolored on addition of the ethanolamine solution. Some ethanolamine is adsorbed on the holo-cellulose (50).


Jayme (51) developed yet another procedure for lignin removal

(51) G. Jayme, Cellulosechemie, 20, 43 (1942).

which involves in situ generation of chlorine dioxide from sodium chlorite in acetic acid solution. Several extractions of 8-12 hr. at 60° were required to reduce the lignin content of wood to 2.8-3.5%.

Wise and co-workers (50) and Whistler and co-workers (52)


modified and greatly shortened the procedure of Jayme. In the
procedure of Whistler and co-workers, fresh portions of sodium chlorite were added to an acidified (pH 4.5) aqueous slurry of ground corn cobs at 15-min. intervals. The reaction was essentially complete in 1 hr. at 75°. The lignin content of the material was reduced to ca. 0.5%.

It was commonly agreed for some time that polysaccharides suffered very little degradation by these mild methods of lignin removal. However, Holmberg and Jahn (53) found a decrease in


pentosan content when holocellulose was prepared by either the chlorine dioxide procedure or by the chlorine-ethanolamine method.

In an investigation of the action of acid chlorite on wheat straw, Harwood (54) isolated polysaccharides containing glucose,

(54) V. D. Harwood, Tappi, 35, 549 (1952).

arabinose, xylose, and glucuronic acid from the chlorite liquors. In this case at least, the degradative action of the chlorite treatment is quite general. Nevertheless, these methods represent the most valuable procedures presently available for the purification of crude cell-wall polysaccharides.
III. EXPERIMENTAL WORK

A. Nature of the Starting Material

The coffee beans used in this investigation were grown by Fazenda Limeira (210,000 trees) which is near the railroad station of Morais Sales, Brazil. The location is in a mountainous district at an altitude of 631 meters. The soil type is Massapê (clayey earth), formed in part by the decomposition of calcareous cretaceous minerals. The classification accorded these beans was "Santos 4's", the mildest or least harsh (taste) of the Brazilian coffees.

B. Isolation and Separation of Sugars in the Green Coffee Bean

1. Preliminary Investigations

   a. Determination of the Moisture Content of Green Coffee Beans

   The moisture content of 50 g. of crushed green Santos coffee beans was determined by distillation with toluene. The water was collected in a graduated receiver. The distillation was continued for 2 hr. at which time the water level had ceased to rise. After cooling to room temperature, the volume of water was 4.7 ml. This corresponds to a moisture content of 9.4% for the crushed beans. It is known that some moisture is lost in the crushing process. Taking this into account, the round figure of 10.0% moisture was considered appropriate and has been used in all of the calculations in Section III. B.
b. Extraction of Green Coffee Beans with 80/20 Ethanol/Water

An amount of 50.0 g. of whole green Santos coffee beans containing no wormy or otherwise discolored beans was poured into 100 ml. of absolute ethanol in a large Pyrex test tube (3.5 x 40 cm.) and frozen by placing in a dry ice-trichloroethylene bath for 15-30 min. The frozen beans and alcohol were poured into a blender and blended for 15 min. An amount of 0.5 g. of calcium carbonate (washed free of alkali) was added at the outset of the blending to neutralize organic acids. The temperature of the mixture at the end of the blending operation was about 40°. The mixture (particle sizes ranging from coarse sand to powder) was poured into a quart Mason jar containing 100 ml. of hot 80% ethanol (by weight) and the blender was rinsed quantitatively with 150 ml. of hot 80% ethanol. Amounts of 20.5 ml. of water and 29.0 ml. of absolute ethanol were added. This brought the volume of 80% ethanol to 400 ml., assuming 10% moisture in the bean.

The mixture was boiled (77-79°) for 2-3 min. by heating in a hot water bath. An amount of 15.0 ml. of absolute ethanol was added to replace that boiled away. The jar was sealed, allowed to cool to room temperature, and stored at 5-10° for 24 hr. before use.

The extract was filtered through Whatman No. 1 filter paper in a Böchner funnel. The filtrate, golden yellow in color, was concentrated by warming under reduced pressure on a water bath (30-35°). Nitrogen was bubbled through the solution to prevent bumping.
The filtrate was placed in a 1000-ml volumetric flask and stored at 5-10°C. The residue was placed in a Soxhlet thimble and extracted for 10 hr. with 80% ethanol. The slightly turbid extract resulting was filtered through Whatman No. 1 filter paper in a Büchner funnel and added to the concentrated original extract contained in the 1000-ml. volumetric flask. The flask was filled to the mark with distilled water and stored at 5-10°C. The solution was turbid when cold, due to the presence of slightly soluble fats and oils.

c. Nonvolatile Solids Content of the 80/20 Ethanol/Water Extract

Two 50.0-ml aliquots of the adjusted extract were freed of ethanol by warming (35-40°C) under reduced pressure and then lyophilized. The flasks were dried in a desiccator over Drierite and weighed (0.504 g. and 0.529 g.). The residues were each extracted with two 25-ml portions of dry ether and filtered through a sintered glass funnel. The ether-insoluble substances were dissolved in distilled water and again lyophilized, dried, and weighed (0.466 g. and 0.448 g., respectively). These values correspond to a water-soluble solids content of 0.696% to 0.932% for the extract.

d. Reducing Power of the 80/20 Ethanol/Water Extract

The water-soluble solids of each aliquot (see previous section) were dissolved in distilled water and adjusted to 50 ml. in volumetric flasks. Five-milliliter portions were removed by pipet and analyzed for reducing power by the method of Somogyi (55). Neither consistent

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nor meaningful results could be obtained because of unknown interfering substances which produced a changing end-point. The interfering substances were probably the polyphenolic structures present.

2. Fractionation of an 80/20 :: Ethanol/Water Extract of Green Coffee Beans (Compare with Chart I and Table 6)

An amount of 500 g. of green coffee beans was finely ground (ca. 80 mesh) in a water-jacketed mill. (The beans were ground in a mill at the laboratories of the Nestlé Co., Marysville, Ohio.) Fifty-gram portions were placed in quart Mason jars and covered with 100 ml. of absolute ethanol. The contents of the jar were boiled 2-3 min. to largely inactivate enzymes and the jars were sealed. The cooled jars and contents were sent to Columbus, Ohio, within a few hours. The following additions were made to each jar: 150 ml. of 80/20 :: ethanol/water, 20 ml. of water and 29 ml. of ethanol (total vol., ca. 300 ml. of 80/20 :: ethanol/water, weight ratio) and 0.5 g. of calcium carbonate. The contents of each jar were boiled for 1-2 min. and the jars were re-sealed. The jars were stored for 70 hr. at 20-30° to effect extraction. The jars were not shaken during this period.

The extract was separated from the ground beans by filtration. The filter cake was washed with one liter of 80/20 :: ethanol/water. The filtrate (ca. 4000 ml.) was concentrated under reduced pressure at 40-45° to ca. 100 ml. The viscous amber-colored residue was dissolved almost completely in 800 ml. of 3/1 :: methanol/ethanol and was allowed to stand for 18 hr. at 25-28°. The above mixture was adjusted to 2000 ml. with absolute methanol; only a trace of
material did not dissolve. Three 25-ml. aliquots of the adjusted (to 2000 ml.) extract were used in the solids determination which employed the sand-vacuum drying method (56). The average of the three values (1.126 g., 1.126 g., and 1.127 g.) was 1.126 g. per 25 ml. aliquot or 90.08 g. of total solids extracted from 500 g. (450 g. dry weight) of green coffee beans. On a dry-weight basis, 20.0% of the green coffee bean was soluble in 80% aqueous ethanol.

An amount of 1900 ml. of the adjusted (to 2000 ml.) extract was added at the top of a 11-12 cm. by 7-9 cm. (diam.) bed of 250 g. of a mixture of 5 parts of Florex XXX (57) to 1 part of Celite No. 535 (58) contained in a 2-liter percolator. The bed had been prewet with one liter of 95% ethanol. When the top of the supernatant liquid reached the top of the adsorbent bed, the addition of 95% ethanol was begun (all developer ratios are volume/volume ratios). After percolation of 5 liters of 95% ethanol through the adsorbent bed, the receiver was changed and the addition of 80/20 ethanol/water was begun. The pressure in the receiver was 19 cm. of mercury. The pressure was reduced to 29 cm. and 6 liters of 80/20 ethanol/water were percolated through the bed in 13.5 hr. The pressure was adjusted

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(57) A Fuller's earth clay produced by the Floridin Co. of Warren, Pa.

(58) A siliceous filter aid produced by the Johns-Manville Co., New York, N. Y.
to 10-11 cm. of mercury and 5.5 liters of 50/50 ethanol/water was percolated through the bed in 24 hr. The color of the effluent was light to medium green. Under full water aspirator pressure, 4 liters of distilled water were percolated through the bed in 53 hr. A volume of 3 liters of 0.1 N hydrochloric acid was percolated through the bed in 72 hr. by intermittent application of full water aspirator vacuum. A volume of 4 liters of 0.5 N hydrochloric acid was percolated through the adsorbent bed in 6 days with intermittent application of full water aspirator vacuum.

a. Investigation of the 95/5 ethanol/water Fraction (Compare with Chart II)

The 95/5 ethanol/water effluent fraction (7 liters) was concentrated at 45-50° to 1600 to 1700 ml. and adjusted to 2000 ml. with absolute ethanol. The solution was golden in color and possessed a strong odor of green coffee beans.

The adjusted (to 2000 ml.) solution of the 95/5 ethanol/water fraction gave a positive Molisch test and a positive ferric chloride test (for phenolic substances, notably chlorogenic acid).

The nonvolatile solids content of the 95/5 ethanol/water fraction was determined by the quartz sand-vacuum drying method (54) using three 20-ml. aliquots of the adjusted (to 2000 ml.) solution. The results were 0.711 g., 0.714 g., and 0.713 g. or an average of 0.713 g. per 20 ml. This corresponded to a total nonvolatile solids content of 71.3 g. for the total 95/5 ethanol/water fraction or 75.1 g. of this fraction per 500 g. (450 g. dry weight) of green coffee beans. On a dry-weight basis, 17.8% of the green coffee bean
appeared in the 95/5 fraction.

(1) Direct Crystallization of Sucrose

Aliquots of 200 ml. each of the adjusted (to 2000 ml.) solution of the 95/5 fraction were placed in four weighed 250-ml. beakers. Crystallization was induced by scratching the walls of the beakers with glass rods; crystallization was allowed to proceed at 25-30° for 48 hr. The supernatant liquors were decanted into four additional weighed 250-ml. beakers. The crystals in the first set of beakers were warmed a few minutes with absolute ethanol (25 ml. in each beaker) and the resulting mixture was stored at 2-4° for 24 hr. The supernatant liquors were decanted into their respective beakers. This process was repeated. The crystals were allowed to dry in air at 25-28° for 3 days. The weights of crude sucrose crystals were 1.96 g., 1.90 g., 1.90 g., and 1.92 g. or a total of 7.68 g. per 800 ml. of the 95/5 fraction or 20.2 g. per 500 g. (450 g. dry weight) of green coffee beans.

Some of the best sucrose crystals were removed from the beakers for the determination of physical constants. The melting point of the crude sucrose crystals was 185-186°. An amount of 0.5222 g. of crude sucrose crystals was dissolved in 10 ml. of distilled water. The solution was very turbid. About 0.1 g. of Darco G-60 (59) activated carbon was added to the solution and the solution was warmed and filtered. The carbon treatment was repeated. The specific rotation

(59) Decolorizing charcoal; a product of Darco Department, Atlas Powder Co., New York, N. Y.
(two-decimeter tube) was \([\alpha]_{D}^{2h} +65.9^\circ\) (c 5.222, water).

An amount of 5.00 g. of crude sucrose crystals was dissolved in 10 ml. of distilled water. An amount of 0.1 g. of Darco G-60 activated carbon was added and the mixture was warmed. The filtered solution had a light green color, so the charcoal treatment was repeated. Still the color remained. Exactly one-half of the solution was concentrated to a thick sirup. The sirup was diluted with a few milliliters of methanol and ethanol was added to induce crystallization. Crystallization was further induced by scratching the sides of the beaker with a glass rod and the sucrose was allowed to crystallize for 18 hr. An amount of 2.17 g. of sucrose was recovered. These crystals had a melting point of 189-190° and a specific rotation of \([\alpha]_{D}^{27} +67.1^\circ\) (c 6.468, water), using a two-decimeter tube. The accepted values for sucrose are: m.p. 188°, \([\alpha]_{D}^{20} +66.53^\circ\) (c 26, water).

(2) Removal and Identification of Caffeine

The supernatant liquor from the direct crystallization of sucrose was concentrated to a thick sirup at 45-50° under reduced pressure and transferred, with the aid of 100 ml. of distilled water, to a continuous liquid-liquid extractor. The aqueous solution was extracted for 20 hr. with ethyl ether (previously stored over mercury). The solvent was removed from the ether extract by warming under reduced pressure. The fatty residue was completely dissolved in 150 ml. of petroleum ether (b.p. 30-60°) and the solution was stored at -25° for several days. On warming to room temperature (ca. 27-28°), all but a small amount of amorphous material redissolved. The amorphous material
was collected on a filter and weighed; yield, 0.34 g. The filtrate was freed of solvent at 45-50° under reduced pressure. The brown fatty residue weighed 4.89 g.

The aqueous solution which was extracted was freed of ether at 45-50° under reduced pressure and was then lyophilized. The residue (weight, 13.7 g.) was a nearly white crystalline material. This material was designated Ether-Insoluble Substances of the Crude Sucrose Mother Liquor, (namely, of the mother liquor resulting from the direct crystallization of sucrose from the 95/5 fraction).

An amount of 5.00 g. of the Ether-Insoluble Substances of the Crude Sucrose Mother Liquor was dissolved in 100 ml. of distilled water and extracted for 20 hr. with benzene in a continuous liquid-liquid extractor. The extract was freed of benzene at 60-70° under reduced pressure. The white crystalline residue (0.51 g.) was dissolved in 10 ml. of 95% ethanol by heating. On cooling to room temperature (30°), white needles of caffeine formed which were recovered by filtration; yield, 0.124 g.; m.p. 234-236°. On further cooling to -25° a second crop of crystals was recovered; yield, 0.305 g.; m.p. 231-235°. Evaporation of the mother liquor gave 0.079 g. of a light brown semicrystalline material.

The aqueous solution, which was extracted, was not further investigated.

(3) Crystallization of Sucrose from De-fatted 95/5 : Ethanol/Water Fraction

A volume of 1000 ml. of the adjusted (to 2000 ml.) solution of the 95/5 : ethanol/water fraction was concentrated at 45-50° under
reduced pressure to a volume of 100-150 ml. This heterogeneous liquid residue was transferred to a continuous liquid-liquid extractor. The mixture was extracted for 2 hr. with benzene. The mixture being extracted changed from a greenish golden color to a bright yellow color with a trace of green. The benzene extract had become a golden color. The extraction was continued an additional 6 hr. with fresh benzene. The aqueous mixture which was extracted, became a clear homogeneous solution; it was heated at 45-48°C under reduced pressure to remove dissolved benzene and was then adjusted to 250 ml. with distilled water. The material in this solution was designated Benzene-Insoluble Substances of the 95/5 Fraction. The combined benzene extracts were adjusted to 250 ml. by similar concentration and dilution. The material in this solution was designated Benzene-Soluble Substances of the 95/5 Fraction.

The nonvolatile solids content of the aqueous solution of the Benzene-Insoluble Substances was determined by the sand-vacuum drying method using three 10-ml. aliquots. The results were 1.010 g., 1.014 g., and 1.012 g., an average value of 1.012 g. per 10-ml. aliquot or 25.3 g. per 250 ml. of solution. This corresponded to 53.3 g. of nonvolatile Benzene-Insoluble Substances per 500 g. (450 g. dry weight) of green coffee beans. On a dry-weight basis, 11.8% of the green coffee bean appeared in the Benzene-Insoluble fraction.

The nonvolatile solids content of the benzene solution of the Benzene-Soluble Substances was determined by the sand-vacuum drying method using three 10-ml. aliquots. The results obtained were
0.330 g., 0.339 g., 0.339 g. for an average value of 0.339 g. per 10-ml. aliquot. A determination of soluble substances was also made by heating three 25-ml. aliquots at 50° under reduced pressure to remove all solvents. The results obtained were 0.882 g., 0.852, and 0.850 g. for an average (discarding the first) of 0.851 g. per 25-ml. aliquot. The two methods are substantially in agreement. By the first method the nonvolatile solids content was 8.48 g. per 250 ml. of solution or 17.8 g. per 500 g. (450 g., dry weight) of green coffee beans. On a dry-weight basis, 3.95% of the green coffee bean appeared in the Benzene-Soluble fraction.

Two 70-ml. aliquots of the adjusted (to 250 ml.) aqueous solution of the Benzene-Insoluble Substances of the 95/5 fraction were lyophilized. The residues were transferred, each with the aid of 15 ml. of absolute methanol, to separate weighed 250-ml. beakers. Crystallization of sucrose was induced by scratching the inner walls of the beakers with glass rods and by the addition of 15 ml. of absolute ethanol to each beaker. The beakers were stored for 24 hr. at 2-4°. The supernatant liquors were decanted into separate weighed 250-ml. beakers. The crystals were covered with 15 ml. of absolute ethanol and stored at 2-4° for 19 hr. The supernatant liquors were decanted into their respective beakers and the crystals were allowed to dry at room temperature. The yields were 1.265 g. and 1.142 g.

The melting point of the crude sucrose crystals was 174-195°. The specific rotation was $[\alpha]_D^{2} = 45.6^\circ$ (c 4.53, water), using a two-decimeter tube. The physical constants indicated that the crystals
were impure. The crystals gave a strong positive ferric chloride test and electrochromatography indicated the presence of sucrose, chlorogenic acid, and a trace of caffeic acid. Calculations based on the assumption that chlorogenic acid \( [\alpha]_D^{38} = -38^\circ \) was the only impurity and that the rotations were additive indicate that the crystals were 79% sucrose and 21% chlorogenic acid. These figures correspond to 6.64 g. per 140 ml., 11.85 g. per 250 ml., and 24.9 g. of sucrose per 500 g. (450 g. dry weight) of green coffee beans. On a dry-weight basis, sucrose constitutes 5.53% of the green coffee bean.

(4) Paper Chromatography of the De-fatted 95/5 Ethanol/Water Fraction

The mother liquors from the crystallization of sucrose from the Benzene-Insoluble Substances of the 95/5 fraction formed an amorphous precipitate on standing. The supernatant liquors were decanted into a 200-ml. volumetric flask and adjusted to volume with 95% ethanol.

The nonvolatile solids content of the adjusted (to 200 ml.) solution was determined by removing solvents at 50-60° under reduced pressure, using two 10-ml. aliquots. The flasks were placed in a vacuum desiccator over phosphorus pentoxide to come to constant weight (3 days). The results were 0.264 g. and 0.266 g. per 10-ml. aliquot or 5.3 g. per 200 ml. of solution.

A third 10-ml. aliquot was freed of solvent at 50-60° under reduced pressure. The residue was dissolved in sufficient distilled water to make a 2% solution. Thirty applications (using a capillary tube) of this solution were made to a 0.75 cm. (diam.) circle the center of which was on a line 7.5 cm. from one end of a 4.5 x 1.4 cm.
strip of Whatman No. 1 filter paper. Sucrose, D-glucose, and D-fructose solutions were applied to other circles on the line in amounts sufficient to make definitive spots with the spray reagent. The chromatogram was developed for 40 hr. by the descending method with 40/19/11 :: 1-butanol/water/ethanol. The paper was tapered at the bottom to facilitate dripping. The resulting chromatogram was air-dried, viewed under ultraviolet light, and then sprayed with 1% (wt./vol.) p-anisidine hydrochloride in water-saturated 1-butanol (Figure 1).

(5) Determination of Chlorogenic Acid by Ultraviolet Adsorption

The residue (0.265 g.) from one of the 10-ml. aliquots from the solids determination of the adjusted (to 200 ml.) supernatant liquors from the crystallization of sucrose from the de-fat 95/5 fraction was transferred to a 1-liter volumetric flask with distilled water. The flask was filled to the mark with distilled water and thoroughly shaken. An amount of 50 ml. of this solution was transferred with a pipet to a 500-ml. volumetric flask and diluted to volume. The optical density of this solution at 324 millimicrons was 0.818 (measured with a Beckmann Model DU Spectrophotometer). This corresponds to 59% chlorogenic acid in the original residue. The per cent of chlorogenic acid was calculated using an $E_{\text{1cm}}^{1\%}$ value of 526 for anhydrous chlorogenic acid (60).

Figure 1. Chromatogram of the Benzene-Insoluble Substances after Crystallization of Sucrose
(Y = yellow; B = brown; FY = faint yellow)
(6) Attempted Isolation of Chlorogenic Acid

A volume of 70 ml. of the adjusted (to 200 ml.) supernatant liquors was concentrated to a thick sirup at 50-55° under reduced pressure. The amount of sirup was increased to about 8 ml. with distilled water. Amounts of 0.2 g. of potassium acetate and 0.5 g. of caffeine were dissolved in the solution by warming. The solution was diluted with 8 ml. of 95% ethanol and stored at 12° (61). After three days only a sirup was obtained. The sirup and supernatant liquor were poured into 1 liter of distilled water. To the water solution was added 40 ml. of saturated aqueous neutral lead acetate (20 g. of lead acetate) and 10 g. of Celite No. 535. The mixture was filtered through a thin asbestos bed in a 15-cm. Böchner funnel. The bright yellow residue (a lead complex of chlorogenic acid) was washed with three 50-ml. portions of distilled water. The damp bed was transferred to a 250-ml. graduated cylinder with the aid of 200 ml. of water. Hydrogen sulfide gas was bubbled through the solution until all of the complex was decomposed and no further lead sulfide formed (about 2 hr.). The mixture was filtered. The filtrate was taken to dryness at 50-55° under reduced pressure. The almost white residue was dried overnight over phosphorus pentoxide in a vacuum desiccator and weighed (yield 0.93 g.). On the basis of an ultraviolet adsorption analysis, 1.12 g. of chlorogenic acid was present in the starting mixture.
The 0.93 g. of chlorogenic acid recovered from the sirup, 0.2 g. of potassium acetate, and 0.5 g. of caffeine were dissolved in 8 ml. of water. An amount of 8 ml. of absolute ethanol was added to the solution and it was stored at 4°. A sirup formed in a short time. The mixture was stored at -25°. In the course of several weeks a white semi-crystalline crust formed at the interface of the sirup and supernatant liquor layers. No crystallization occurred.

(7) Reaction of Chlorogenic Acid with a Basic Resin

An amount of 10 g. of ether- and benzene-insoluble material from the 95/5 : ethanol/water fraction of green coffee bean (containing mostly sucrose and chlorogenic acid) was dissolved in 130 ml. of distilled water (light yellow solution) and passed through a column of Duolite A-4 (62). An amount of 500 ml. of distilled water was passed through the column at fast drop rate. The effluent was very dark brown. The lyophilized effluent material (4.9 g.) was also dark brown and still produced a precipitate with neutral lead acetate. The resin was washed with 500 ml. of 10% sodium hydroxide. The effluent was practically black. The effluent was neutralized immediately with hydrochloric acid and the color changed to dark red-brown. The material was not further investigated.

(8) Preparation of a Concentrate of Trace Sugars

A volume of 100 ml. of the adjusted (to 200 ml.) supernatant liquors from the crystallization of sucrose from de-fatted 95/5
fraction was concentrated to a sirup at 50-55°. The sirup was dis-
solved in water and diluted to a volume of 48 ml. About 2 ml. of
saturated neutral lead acetate solution (1 g. of lead acetate) was
added. The yellow precipitate which formed was removed by filtra-
tion. The residue was transferred to a 250-ml. graduated cylinder
with the aid of 100 ml. of water and decomposed with hydrogen sulfide.
The filtrate was similarly freed of lead. Lead sulfide was removed
by filtration. Both final filtrates, one containing substances pre-
cipitated by lead ions and the other containing substances not pre-
cipitated by lead ions, were neutralized to the color change of
chlorogenic acid with potassium carbonate and then lyophilized. The
lead-precipitated material (presumably the potassium salt of chloro-
genic acid) was dark yellow. The material not precipitated by lead
tended to be sirupy and was also yellow, indicating that not all of
the chlorogenic acid had been removed.

An amount of 1.0 g. of the sirup was twice more treated with
lead acetate. The final lyophilized material was a translucent,
exceptionally hygroscopic sirup (weight, about 0.77 g.) but was no
longer yellow. The sirup gave a moderately strong Molisch test and
a weak ninhydrin test. The sirup contained considerable ash.

Paper chromatograms (Figure 2) (made by the method described
under Paper Chromatography of the Mother Liquor from the Crystalliza-
tion of Sucrose from the Benzene-Insoluble Substances of the 95/5
Fraction) of the sirup were very interesting in that they failed to
confirm the presence of glucose as indicated by earlier chromatograms
(Figure 1). This may have been due to loss by adsorption on the
Figure 2. Chromatogram of the Benzene-Insoluble Substances Not Precipitated by Lead
(Y = yellow)
b. Investigation of the 80/20 :: Ethanol/ Water Fraction

The 6 liters of 80/20 :: ethanol/water fraction effluent were concentrated to ca. 130 ml. under reduced pressure at 45-50°. The solution was murky. The addition of this solution to 5 volumes of absolute methanol caused a green precipitate to form. The precipitate was collected on a 4.25 cm. filter. It was dark green; the precipitate weighed 100 mg.

The aqueous methanol solution was concentrated under reduced pressure at 45-50° to a volume slightly less than 100 ml. This concentrate was adjusted to 100 ml. with distilled water. The nonvolatile solids content of the adjusted solution was determined by the sand-vacuum drying method, using three 10-ml. aliquots. The results obtained were 1.000 g., 1.000 g., and 1.004 g. or an average value of 1.00 g. per 10-ml. aliquot. This corresponded to a total nonvolatile solids content of 10.0 g. for the 100 ml. solution or 10.5 g. of this fraction per 500 g. (450 g. dry weight) of green coffee beans. On a dry-weight basis, 2.33% of the green coffee bean appeared in the 80/20 fraction.

Some of the solids of the 80/20 :: ethanol/water fraction were recovered from the sand with distilled water. The resulting solution was lyophilized. An amount of 60 ml. of the adjusted (to 100 ml.) solution was lyophilized.

A 10-ml. aliquot of the adjusted solution was used for qualitative tests and paper chromatography. The solution gave a positive
Molisch test, a strong positive ferric chloride test, and a positive ninhydrin test.

An amount of 200 mg. of the lyophilised solids of the 80/20 fraction was analysed for chlorogenic acid content by the ultraviolet adsorption method (60). The measured optical density of the prepared test solution was 0.458 at 324 millimicrons. This corresponds to a chlorogenic acid content of 4.6% for the solids of the 80/20 fraction or 4.6 g.

(1) Paper Chromatography of the 80/20 Ethanol/Water Fraction

The material of the 80/20 fraction was chromatographed as usual beside known sucrose, D-glucose, and myo-inositol. The chromatogram was developed for 45.5 hr. It was viewed under ultraviolet light and sprayed with 1% (wt./vol.) p-anisidine in water-saturated l-butanol and with 0.05 M aqueous sodium metaperiodate followed by 1% aqueous potassium permanganate (Figure 3).

The material of the 80/20 fraction was also chromatographed on large sheets of filter paper. An amount of 26 mg. of adsorbent was added in a streak near one side of a 47 x 47 cm. sheet of Whatman No. 1 filter paper. The chromatogram was developed for 27-28 hr. with 40/19/11 l-butanol/water/ethanol. Four such chromatograms were prepared. The chromatograms were viewed under ultraviolet light (Figure 4). Zone 2 was eluted from the chromatograms with water. The light brown, amorphous material gave a green color with ferric chloride solution and was probably mainly chlorogenic acid. Because of the small amount, no attempt was made to crystallize the material.
Figure 3. Chromatogram of the 80/20 Ethanol/Water Fraction (Y = yellow)
Figure 4. Chromatogram of the 80/20 Fraction on a Filter Paper Sheet
(2) Electrochromatography of the 80/20 EthanclA^ater Fraction

The technical and theoretical principles of electrochromatography can be simply stated as follows.

The electrical apparatus consists of a 1000-1200 volt direct current power supply. The high voltage leads are attached to the electrodes of two plastic tanks containing borate buffer of pH 10.

The material to be chromatographed is applied to a strip of filter paper as usual but at a point on a line near the middle of the strip. The filter paper is moistened carefully with the buffer solution and clamped between two glass plates somewhat wider and shorter than the paper strip. The plates are supported so that the extended ends of the paper dip into the two tanks. The moist paper is a high resistance load in the high voltage circuit.

When a potential is applied to the strip, the adsorbed material moves toward the positive or negative electrode depending on its charge in the buffered solution. Cations migrate along the paper toward the anode, while anions and substances which form negatively charged complexes with borate ions (such as vicinal diols) migrate toward the cathode. Uncharged particles follow, and the charged particles are displaced by the electrophoretic flow of the solvent toward the anode. The fact that various sugars complex with borate ion to an extent dependent upon their configurations makes this method a valuable tool in sugar research.

Appropriate amounts of the 80/20 fraction, the 95/5 fraction, and trigonelline were electrochromatographed. The chromatogram was viewed
under ultraviolet light (Figure 5).

(3) Identification of Amino Acids

Employing the two-dimensional, ascending method with 80% aqueous phenol and water-saturated 1/1: collidine/lutidine developers used by Kowkabany, Binkley, and Wolfrom (63), the following amino acids were identified in the 80/20: ethanol/water fraction: glycine, \( \alpha \)-alanine, \( \gamma \)-aminobutyric acid, proline, valine, leucine, and tyrosine. Glycine, \( \alpha \)-alanine, \( \gamma \)-aminobutyric acid, and valine were identified by position and color of spots; proline, leucine, and tyrosine were confirmed by spot enhancement with known compounds. \( \alpha \)-Alanine and \( \gamma \)-aminobutyric acid were estimated to constitute one-half of the total weight of amino acids present by comparison of spot intensity with knowns.

(4) Isolation of Chlorogenic Acid

Amounts of 4.6 g. of solids of the 80/20 fraction, 1.1 g. of caffeine, and 0.22 g. of potassium acetate were dissolved in 12 ml. of water by heating. The solution was brown and viscous. An amount of 12 ml. of 95% ethanol was added and the solution was stored at 1° for three days. The light brown, semicrystalline precipitate (crude potassium caffeine chlorogenic complex) was removed by filtration. It was dissolved in a small amount of hot water and precipitation was initiated by the addition of a small amount of 95% ethanol. The
Figure 5. Electrochromatogram of the 80/20 and 95/5 Fraction
solution was stored at 1° for 20 hr.

The recrystallized complex was removed by filtration and dried at room temperature. The filtrates were combined and stored at -25°. The complex was dissolved in 8-9 ml. of water containing 0.8 g. of tartaric acid. The solution was cooled and the potassium bitartrate (brown, semicrystalline mass) was removed by filtration. The filtrate was diluted to twice its original volume (about 18 ml.) and extracted for 24 hr. with chloroform in a continuous liquid-liquid extractor. The extracted aqueous solution was concentrated to about 10 ml. and stored at 1°. A few white tablets formed on the sides of the extraction apparatus above the water level during the extraction. These crystals were recovered and dried over phosphorus pentoxide in a vacuum desiccator (yield 0.026 g.; m.p. 180-195° with some frothing). Light yellow plates formed in the solution stored at 1°. These crystals were likewise dried over phosphorus pentoxide (yield 0.450 g.; m.p. 185-195° with much frothing). A second crop of light yellow tablets was obtained by concentrating the mother liquor and storing overnight at 1°. These crystals were dried over phosphorus pentoxide and weighed (yield 0.332 g.; m.p. 193-194° with little frothing). The frothing at the melting point was found to be caused by tartrate or tartaric acid impurity by melting known pure chlorogenic acid mixed with a small amount of tartaric acid.

The second crop of crude chlorogenic acid crystals (m.p. 193-194°) was recrystallized three times from water. An amount of 0.048 g. of white spherulitic clusters was recovered, m.p. 203°,
\([\alpha]_D^{26} -47 \pm 7^\circ (c 0.2, \text{water})\). The constants for pure chlorogenic acid are: m.p. 206-208\(^\circ\), \([\alpha]_D -38^\circ\).

c. Investigation of the 50/50 :: Ethanol/Water Fraction

The 6 liters of the 50/50 fraction effluent were concentrated to slightly less than 100 ml. at 48-50\(^\circ\) under reduced pressure. The concentrate was adjusted to 100 ml. with distilled water. The nonvolatile solids content of the 50/50 fraction was determined by the sand-vacuum drying method on three 10-ml. aliquots of the adjusted solution. The results were 0.243 g., 0.242 g., and 0.242 g., or an average value of 0.242 g. per 10-ml. aliquot. This corresponds to a total nonvolatile solids content of 2.55 g. per 500 g. (450 g. dry weight) of green coffee beans. On a dry-weight basis, 0.57% of the green coffee bean appeared in the 50/50 fraction. An amount of 60 ml. of the adjusted solution was lyophilized. The material had a great tendency to thaw and required re-freezing. Although some thawing did occur, the drying was finally accomplished. The dry material was dark green in color. One 10-ml. aliquot was used for qualitative tests and paper chromatography.

(1) Paper Chromatography of the 50/50 :: Ethanol/Water Fraction

Descending paper chromatography using 40/19/11 :: 1-butanol/water/ethanol as the developer indicated the presence of trigonelline and several unidentified substances which gave red-purple spots with ninhydrin reagent (later identified, see following section). One unidentified spot was present that gave a yellow-brown (carbohydrate)
color with p-anisidine hydrochloride. The chromatogram showed poor resolution.

Electrochromatography confirmed the presence of trigonelline in the 50/50 fraction.

d. Identification of Amino Acids

Using the method of Binkley, Kowkabany, and Wolfrom (63), the following amino acids were identified in the 50/50 ethanol/water fraction: aspartic acid, glutamic acid, serine, asparagine, \( \alpha \)-alanine, and \( \gamma \)-aminobutyric acid. Aspartic acid, glutamic acid, serine, and asparagine were identified by position and color of spots; \( \alpha \)-alanine and \( \gamma \)-aminobutyric acid were confirmed by spot enhancement with known compounds. Only trace amounts of \( \alpha \)-alanine and \( \gamma \)-aminobutyric acid were present in this fraction.

e. Investigation of the Water Fraction

The 4 liters of the water fraction effluent were concentrated to slightly less than 100 ml. in volume under reduced pressure at 48-50°C. The concentrate was adjusted to 100 ml. with distilled water. The nonvolatile solids content of the solution was determined by the sand-vacuum drying method using three 10-ml. aliquots. The results were 0.126 g., 0.126 g., and 0.126 g., an average value of 0.126 g. per 10-ml. aliquot or 1.26 g. per 100 ml. This corresponded to 1.32 g. of water fraction per 500 g. (450 g. dry weight) of green coffee beans. On a dry-weight basis, 0.30% of the green coffee bean appeared in the water fraction.

Paper chromatography of the water fraction by the descending
method produced a slow-moving spot which had a bright green fluorescence under ultraviolet light and gave a purple color with ninhydrin reagent. Another slower moving spot gave a dark brown color with p-anisidine hydrochloride that required several days for color formation. The chromatogram was crowded and poorly resolved.

An amount of 60 ml. of the adjusted (to 100 ml.) solution of the water fraction was lyophilized. The residue was dark brown and resembled "browning polymer" in appearance.

f. The Acid Eluent Fractions

The 3 liters of the 0.1 N hydrochloric acid fraction were light green in color. The fraction was neutralized with silver carbonate. The precipitated silver chloride was collected on a suction filter. The filtrate was saturated with hydrogen sulfide. A small amount of brown silver sulfide precipitated and was removed by filtration. The filtrate was stored at 25-30° under toluene.

The 4 liters of 0.5 N hydrochloric acid fraction were medium green in color. The fraction was stored under toluene at 25-30°. The solids content of these two fractions was calculated by difference to be 0.6 g.

g. Methods for Detecting Caffeine on Paper Chromatograms and Clay Columns

The paper chromatogram (developed as usual or electrochromatographed) is dried and then dipped in a 0.01% solution of pyrene in ethyl ether which has been acidified with a few drops of concentrated hydrochloric acid (degree of acidification not critical). The paper dries in a few seconds and is then viewed under ultraviolet light.
The caffeine spot is observed as an area of quenched fluorescence on a bright yellow-white fluorescent background. The limit of sensitivity is about 20% of caffeine in a 15 mm. diameter spot. It has been found (64) that a large number of purines quench the


fluorescence of acidified aqueous solutions of various polynuclear aromatic substances.

Caffeine was detected on dried clay columns by streaking with very slightly acidified bromothymol blue indicator. The caffeine zone appeared as a blue band on a yellow streak. The acidification of the indicator with a strong acid is critical for caffeine is a weak base; its solutions do not even affect the color of litmus. A possible explanation of the color change when acidified indicator is used, is that caffeine forms the caffeine salt of the strong acid thus reducing the hydrogen ion concentration sufficiently to cause the color change.

h. Isolation of Isochlorogenic Acid

One kilogram of ground (impact mill) green coffee beans and 6 liters of 70% isopropyl alcohol were stirred for 3 hr. at room temperature. The mixture was filtered. The filter cake was stirred 15 min. with another 6-liter portion of 70% isopropyl alcohol and filtered. The combined filtrates were concentrated under reduced pressure to about 2 liters. After standing in the icebox for several days, the concentrate was filtered through Celite No. 535 (58) to
remove fats and waxes. The solution was acidified to pH 2.5 with sulfuric acid and extracted with three 1-liter portions of butyl acetate. The combined extracts, washed with three 250-ml. portions of distilled water, were concentrated until solids appeared. The crude acid was precipitated at this point by the addition of 10-20 volumes of chloroform, filtered, and dried over phosphorus pentoxide (yield, 6.0 grams). This isolation follows closely the method of Barnes, Feldman, and White (65).


The crude acid was found by paper chromatography to be about 50% chlorogenic acid. The crude acid was dissolved in 1 liter of warm water and extracted with three 300-ml. portions of butyl acetate. The combined extracts were evaporated to dryness under reduced pressure and stored in a desiccator over phosphorus pentoxide. This product (2.5 g.) was practically free of chlorogenic acid (see Figure 6) and was extremely hygroscopic.

C. Investigation of the Sugars of Roast Coffee Bean

1. Determination of the Moisture Content of Roast Coffee Bean

The moisture content of 50 g. of finely ground roast Santos coffee beans was determined by distillation with toluene. The water was collected in a graduated receiver. The distillation was continued until the water level in the receiver had ceased to rise. After cooling to room temperature, the volume of water was 2.7 ml.
Adorbate

$G = \text{green}$

$R = \text{roast}$

ICA = isochlorogenic acid

Chlorogenic acid

Not identified

Spot enhancement with isochlorogenic acid

Not identified

Figure 6. Chromatogram of isochlorogenic Acid and Coffee Fractions
This corresponds to a moisture content of 5.4% for the ground roast bean employed in this investigation. This value has been used in placing all subsequent percentages in Section III. C. on a dry-weight basis.

2. Extraction of Roast Coffee Bean with 60/20 : Ethanol/Water

Five hundred grams of finely ground roast coffee beans were shaken with 1 liter of petroleum ether (b.p. 30-60°) and let stand 15 min. The mixture was filtered through Whatman No. 1 filter paper in a large Büchner funnel. The filtrate was concentrated during the filtration and was stored at -25°. The filter cake was placed in a 1-gallon jar and covered with 3 liters of an 60/20 : ethanol/water mixture. The jar was tightly sealed and kept in the dark at 25-35° for 6 days; the jar was shaken occasionally.

3. Fractionation of an 60/20 : Ethanol/Water Extract of Roast Coffee Bean (Compare with Chart III and Table 7)

The extract was separated from the ground bean by filtration. The filter cake was washed with 1 liter of 60/20 : ethanol/water. The filtrate (ca. 4000 ml.) was concentrated under reduced pressure at 37-43° to ca. 200 ml. The solution was further concentrated to ca. 100 ml. by lyophilization. The dark red-brown sirupy residue was dissolved in 800 ml. of 3/1 : methanol/ethanol and adjusted to 2000 ml. with absolute methanol. A 100-ml. aliquot of this solution was set aside for the nonvolatile solids determination. The nonvolatile solids content was determined by the quartz sand-vacuum drying method. The average of three values (1.000 g., 0.998 g.,...
and 1.002 g.) was 1.000 g. per 25-ml. aliquot, corresponding to 80.00 g. of total solids extracted from 500 g. (473 g. dry weight) of roast coffee bean. On a dry-weight basis, 16.93% of the roast coffee bean was soluble in 80/20 :: ethanol/water.

An amount of 1900 ml. of the adjusted (to 2000 ml.) extract was added at the top of a 11-12 by 7-9 cm. bed of 250 g. of a mixture of 5 parts of Florex XXX (57) to 1 part of Celite No. 535 (58) (wt. ratio) contained in a 2-liter pharmaceutical percolator. The adsorbent was prewashed with 1 liter of 95% ethanol. When the top of the supernatant liquid reached the top of the adsorbent bed, the addition of 95/5 :: ethanol/water was begun (all developer ratios are volume/volume ratios). After the percolation of 5 liters of 95/5 :: ethanol/water through the adsorbent column, the receiver was changed and the addition of 80/20 :: ethanol/water was begun. The 1900 ml. of adjusted extract plus 5 liters of 95/5 :: ethanol/water passed through the column in 7.5 hr. The pressure in the receiver was 32 cm. of mercury. At the same pressure 6 liters of 80/20 :: ethanol/water passed through the column in 8 hr. The receiver was changed and the addition of 50/50 :: ethanol/water was begun. The pressure in the receiver was reduced to 24 cm. of mercury. Five liters of 50/50 :: ethanol/water passed through the column in 8 hr. The receiver was changed and the addition of water was begun. Four liters of water passed through the column in 15 hr. The receiver was changed and the addition of 0.1 N hydrochloric acid was begun. A volume of 6 liters of 0.1 N hydrochloric acid passed through the column in 6
days. The effluent was collected under reduced pressure; full
water aspirator vacuum was applied intermittently to the receiver.
The 0.1 N hydrochloric acid eluate was light yellow-brown. All of
the other eluates were dark brown.

a. Investigation of the 95/5: Ethanol/Water Fraction

The 7-liter 95/5: ethanol/water fraction effluent was con­
centrated under reduced pressure at 37-54° to ca. 1700 ml. The
concentrate was adjusted to 2000 ml. with absolute ethanol. A
volume of 100 ml. was set aside for analysis; 1000 ml. was con­
centrated and lyophilized; 800 ml. was used for attempted sucrose
crystallization.

The nonvolatile solids content of the 95/5: ethanol/water
fraction was determined by the sand-vacuum drying method using three
25-ml. aliquots. The results were 0.710 g., 0.713 g., and 0.707 g.
or an average of 0.710 g. per 25 ml. This corresponded to a total
nonvolatile solids content of 56.80 g. for the 95/5: ethanol/
water fraction or 59.64 g. of this fraction per 500 g. (473 g. dry
weight) of roast coffee bean. On a dry-weight basis 12.61% of the
roast coffee bean appeared in the 95/5 fraction.

Aliquots of 200 ml. each of the adjusted (to 2000 ml.) solution
of the 95/5 fraction were placed in four weighed 250-ml. beakers.
The sides of the beakers were scratched occasionally to induce
crystallization but no crystals formed on standing 6 weeks at room
temperature. A dark brown, amorphous precipitate formed but was not
investigated.
A paper chromatogram of the original extract of roast coffee bean, the 95/5 fraction, and known sucrose, D-glucose, and D-fructose was prepared. The chromatogram was developed 40 hr. by the descending method with 40/19/11 1-butanol/water/ethanol. The chromatogram was air-dried, viewed under ultraviolet light, and then sprayed with 1% (wt./vol.) p-anisidine hydrochloride in 1-butanol (Figure 7).

The presence of isochlorogenic acid or caffeic acid or both in the 95/5 ethanol/water fraction was indicated by the two chromatograms, Figure 6 and Figure 8. These chromatograms were developed 17 hr. with 40/19/11 1-butanol/water/ethanol and viewed under ultraviolet light.

The presence of caffeine in the 95/5 ethanol/water fraction was shown by comparative chromatography with known caffeine and 95/5 ethanol/water fraction from green coffee bean (Figure 9). The chromatogram was developed 11 hr. with 40/19/11 1-butanol/water/ethanol. The chromatogram was viewed under ultraviolet light and then dipped in acidified 0.01% pyrene in ether and viewed under ultraviolet light for the detection of caffeine.

b. Investigation of the 80/20 Ethanol/Water Fraction

The 6-liter 80/20 ethanol/water fraction effluent was concentrated under reduced pressure at 37-48° to ca. 500 ml. The concentrate, containing some black, undissolved material, was lyophilized to ca. 75 ml. The solution was adjusted to 200 ml. with distilled water.
Figure 7. Chromatogram of Roast Coffee Bean Extract and 95/5 Fraction
Adsorbate

G = green
R = roast
CA = caffeic acid

Chlorogenic acid

Not identified

Spot enhancement with caffeic acid

Not identified

Figure 8. Chromatogram of Caffeic Acid and Coffee Fractions
Figure 9. Chromatogram of Caffeine, Trigonelline, and Coffee Fractions
The nonvolatile solids content of the 80/20 fraction was determined by the sand-vacuum drying method, using three 10-ml aliquots. The results were 0.544 g, 0.538 g, and 0.537 g, an average of 0.540 g per 10-ml aliquot. This corresponded to a total nonvolatile solids content of 10.80 g for the 80/20 fraction effluent, or 11.34 g of this fraction per 500 g (473 g dry weight) of roast coffee bean. On a dry-weight basis 2.40% of the roast coffee bean appeared in the 80/20 ethanol/water fraction.

A chromatogram of the 80/20 fraction together with the other isolated fractions was prepared. The chromatogram was developed for 40 hr. with 40/19/11 1-butanol/water/ethanol. The chromatogram was viewed under ultraviolet light and sprayed with 1% p-anisidine hydrochloride in 1-butanol (Figure 10).

The presence of isochlorogenic acid or caffeic acid or both in the 80/20 ethanol/water fraction was indicated by the two chromatograms, Figure 6 and Figure 8.

The presence of trigonelline in the 80/20 ethanol/water fraction was indicated by a chromatogram, Figure 9.

No amino acids were found in the 80/20 ethanol/water fraction of roast coffee bean by the method (61) used to identify the amino acids in the 80/20 ethanol/water fraction of green coffee bean.

c. Investigation of the 50/50 Ethanol/Water Fraction

The 5-liter 50/50 ethanol/water fraction effluent was concentrated under reduced pressure at 37-48° to ca. 900 ml. The 900 ml of concentrate was lyophilized to ca. 75 ml. The volume of
Adsorbate

Yellow fluorescent streaks

Quenched uv

Sucrose

Glucose

Fructose

Chlorogenic acid

Figure 10. Chromatogram of the Roast Coffee Fractions
the solution was adjusted to 200 ml. with distilled water.

Three 10-ml. aliquots of the adjusted (to 200 ml.) solution were used in the solids determination by the sand-vacuum drying method. The results were 0.286 g., 0.283 g., and 0.281 g., an average of 0.283 g. per 10-ml. aliquot. This corresponded to a total of 5.66 g. of 50/50 fraction, or 5.94 g. of this fraction per 500 g. (473 g. dry weight) of roast coffee bean. On a dry-weight basis 1.26% of the roast coffee bean appeared in the 50/50 ethanol/water fraction.

The investigation of this fraction was limited to the chromatogram represented by Figure 10.

No amino acids were found in the 50/50 ethanol/water fraction of roast coffee bean by the method (61) used to identify the amino acids in the 50/50 ethanol/water fraction of green coffee bean.

d. Investigation of the Water Fraction

The ½-liter water fraction effluent was concentrated under reduced pressure at 37-48° to ca. 900 ml. The 900 ml. of concentrate was lyophilized to ca. 50 ml. The volume was adjusted to 100 ml. with distilled water.

Two 10-ml. aliquots of the adjusted (to 100 ml.) solution were used in the solids determination by the sand-vacuum drying method. The results were 0.187 g. and 0.183 g., an average of 0.185 g. per 10-ml. aliquot. This corresponded to 1.85 g. of nonvolatile solids in the water fraction or 1.94 g. of this fraction per 500 g. (473 g. dry weight) of roast coffee bean.

The investigation of this fraction was limited to the
chromatogram represented by Figure 10.

e. The Acid Eluate Fraction

The 6-liter 0.1 N hydrochloric acid fraction effluent was stored under toluene in amber bottles at room temperature. By difference this fraction amounted to 1.08 g. per 500 g. (473 g. dry weight) of roast coffee bean. On a dry-weight basis, 0.22% of the roast coffee bean appeared in the 0.1 N hydrochloric acid fraction.

D. Isolation and Investigation of the Polysaccharide Components of the Green Coffee Bean (Compare with Chart IV)

1. Preliminary Treatment of the Starting Material

Green Santos coffee beans, containing no wormy or otherwise discolored beans or foreign matter, were finely ground. Amounts of 600.0 g. of the ground coffee were weighed into each of ten 2-quart Mason jars. To each jar was added 600 ml. of abs. ethanol. The contents of the jars were boiled for 3-5 min. (ca. 80°).

The moisture content of the ground green coffee was 7% as determined by drying at 100° for 2 hr. (66).

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(66) Kindly determined by The Nestlé Co. Research Laboratory, Marysville, Ohio.

The nonvolatile solids content of one 2-quart Mason jar (600-g. amount) was determined by allowing the contents to dry in air. After 6 days the coffee weighed 611.3 g. The moisture content was determined (1) by heating 1-g. samples in a vacuum oven at 70° for 16 hr. and (2) by distillation of a 50.0-g. sample in toluene for 2 hr. The values obtained were 4.9% and 5.6%, respectively (calculated for the
original 600-g. amount). The latter value was used in subsequent calculations.

2. Extraction with 80/20 : : Ethanol/Water

An amount of 2400 g. (2266 g., dry wt.) of the prepared ground green coffee was placed in a 5-gal. bottle. Amounts of 2180 ml. of distilled water and 11,550 ml. of absolute ethanol were added to the bottle. This brought the composition of the solvent to approximately 80/20 : : ethanol/water (wt. ratio). The mixture was kept at 25-30° for 72 hr. and was shaken occasionally during that time.

The mixture was filtered through coarse filter paper in a large table-top Bächner funnel. The residue was washed with 3.5 liters of 80/20 : : ethanol/water and 500 ml. of 95% ethanol.

The combined filtrates and wash liquors (ca. 16 liters) were concentrated to ca. 2 liters in a laboratory flash evaporator (67).

(67) Catalog No. 07965, The Emil Greiner Co., 20-26 N. Moore St., New York, N. Y.

The concentrate was further reduced in volume to ca. 700 ml. under reduced pressure at 45-50° on a water bath. The resulting mixture of brown solids, fats, and water was placed in a continuous extractor and extracted for 16 hr. with petroleum ether (b.p. 30-60°). The solvent was removed under reduced pressure at 50°, yielding 58.0 g. of a brown oil.

The aqueous layer from the petroleum ether extraction was next extracted for 10 hr. with ethyl acetate. On removal of the solvent under reduced pressure at 50°, 9.0 g. of red-brown resin was obtained.
The aqueous layer from the ethyl acetate extraction was lyophilized. A cream-colored powder (263.3 g.) was obtained. The total 80/20 :: ethanol/water extract constituted 15.5% of the original bean on a dry-weight basis.

3. Extraction with 2/1 :: Benzene/Ethanol

The residue (not dried) from the 80/20 :: ethanol/water extraction of 2400 g. of ground green coffee was placed in a modified Soxhlet extractor and extracted with 2/1 :: benzene/ethanol (approx. the azeotrope). The extraction was continued for a time (ca. 20 hr.) sufficient for 50 or more changes of solvent to occur.

The 2/1 :: benzene/ethanol extract (ca. 5 liters) contained some insoluble yellow solids. Amounts of 2 liters of benzene and 10 liters of ethanol were added to make a homogeneous solution (17.25 liters). Two 10-ml. aliquots of the adjusted solution were used in the determination of the nonvolatile solids by the quartz sand-vacuum drying method. The values obtained were 0.223 g. and 0.224 g. or an average of 0.224 g. per 10-ml. aliquot. This corresponded to a total of 386 g. of material in the 2/1 :: benzene/ethanol extract or 17.1% of the original bean on a dry-weight basis.

The residue from the benzene/ethanol extraction was dried in air at 25-30° until only a trace odor of ethanol remained (6 days); yield, 1751 g. A nonvolatile solids determination was carried out on three 5.000-g. samples of the residue by drying in a vacuum oven at 70° for 16 hr. The values obtained were 4.327 g., 4.323 g., and 4.318 g. Using an average value of 4.323 g., the dry weight of the
residue amounted to 151/4 g. This residue constituted 66.8% of the original bean on a dry-weight basis.

The residual oil in a sample of ground green coffee which had been defatted by the benzene/ethanol procedure was determined by exhaustive extraction with ethyl ether and petroleum ether (66). The values obtained were 0.43% by a 72-hr. extraction with ethyl ether and 0.48% by a 72-hr. extraction with petroleum ether.

1. Extraction with Water

An amount of 1388 g. (dry wt.) of residue from the benzene/ethanol extraction was placed in a 5-gal. bottle and covered with 8 liters of distilled water. The contents were shaken for 30 min. at room temperature. The mixture was filtered through a layer of milk filter-disks (68) and washed with distilled water (14 liters) until the effluent changed from brown to grey in color. The residue was stored at -25°.

The filtrate (9.5 liters, neutral to litmus) was poured with stirring into 22 liters of absolute ethanol (final conc., 70% ethanol). The flocculent cream-colored precipitate which formed was recovered by filtration through coarse filter paper in a large Bürchner funnel. The precipitate was dried by suspending in water and lyophilizing; yield, 72.7 g. of grey powder (3.8% ash as sulfate). This material constituted 3.5% of the original bean on a dry-weight basis. The 70% alcohol-insoluble, grey powder gave a negative ferric
chloride test (for phenols), a positive ninhydrin (blue) test, and a positive Molisch test.

A 250-mg. sample of the 70% alcohol-insoluble material was refluxed in 25 ml. of N sulfuric acid for 6 hr. (homogeneous solution). The hydrolyzate was neutralized with barium carbonate and filtered. Chromatography of the filtrate on paper using 40/19/11 :: 1-butanol/water/ethanol (vol. ratio) as the developer and p-anisidine hydrochloride as the spray reagent, showed the presence of arabinose and galactose.

The filtrate from the 70% ethanol precipitation was concentrated (67) and lyophilized. An amount of 213.0 g. of dense yellow solids was obtained. This amounted to 10.2% of the original bean on a dry-weight basis.

A second water extraction was carried out by placing the residue from the first extraction in 5.5 liters of distilled water in a 5-gal. bottle and shaking occasionally for 1 hr. at 15-20°. The mixture was again filtered through milk filter-disks (68). The residue was washed with 4 liters of distilled water and stored at -25°.

The filtrate (9.7 liters, neutral to litmus) was concentrated (67) to 1 liter and then lyophilized; yield, 21.8 g. of fluffy tan solids (1.1% of original bean, dry-weight basis; ash, 16.3 and 16.9%, as sulfate). This material gave a strongly positive (green) ferric chloride test, a strong positive ninhydrin test (purple), and a strong positive Molisch test.

A third water extraction was carried out by placing the residue from the second extraction in 5.7 liters of distilled water in a
5-gal. bottle. The mixture was kept at 15-20° for 2 hr. and occasionally shaken. The mixture was filtered through milk filter-disks (66). The residue (1065 g., dry wt.) was stored at -25°. This residue constituted 51.3% of the original bean on a dry-weight basis.

The filtrate (ca. 9.5-10.0 liters, neutral to litmus) was concentrated (67) to 1 liter and lyophilized; yield, 8.7 g. of fluffy, light-green solids (0.4% of original bean, dry-weight basis; ash, 1.01 and 1.11%, as sulfate). This material gave a positive (green, catechol) ferric chloride test, a positive ninhydrin test (bluish-purple), and a weak positive Molisch test.

5. Extraction with Dilute Ammonium Oxalate

An amount of 376 g. (dry wt.) of residue from the third water extraction was added to 13 liters of distilled water containing 60 g. of ammonium oxalate (slightly less than 0.5% conc.) at 90°. The mixture was vigorously stirred and kept at 90 ±2° for 1 hr. The mixture was rapidly filtered through milk filter-disks (68) and the residue was washed with 4 liters of distilled water. The residue was stored at -25°. The residue gave a negative ferric chloride test, a strongly positive ninhydrin test, and a positive Molisch test.

The filtrate (ca. 17 liters, neutral to litmus) was concentrated (67) to slightly less than 2 liters and then lyophilized; yield, 77.3 g. of fluffy, white solids (ash, 3.67 and 3.82% as sulfate). This material gave a negative ferric chloride test, a strongly positive ninhydrin test, and a positive Molisch test.
The residue from the first ammonium oxalate extraction was extracted with 12 liters of 0.5% ammonium oxalate solution at 90±3°C for 1 hr. and filtered. The residue was stored at -25°C. The filtrate was concentrated (67) to less than 2 liters and lyophilized; yield, 63.6 g. of fluffy, white solids (ash, 0.66 and 0.61%, as sulfate). This material gave a negative ferric chloride test, a strongly positive ninhydrin test, and a positive Molisch test.

The dry weight of the insoluble residue was 34.27 g. This constituted 46.8% of the original bean on a dry-weight basis.

The ash content of the residue from the second ammonium oxalate extraction was determined (0.19 and 0.15% ash, as sulfate). Assuming the metal to be calcium, this ash value indicates that the residue could contain no more than 0.6 g. of calcium oxalate. Thus, practically all of the ammonium oxalate (120 g.) used in the extractions must have gone into the filtrate unchanged. Therefore, the amount of 119.5 g. is subtracted from the weight of the lyophilized ammonium oxalate extracts to obtain the weight (21.4 g.) of material (pectin) extracted. This material amounted to 3.0% of the original bean on a dry-weight basis.

An amount of 0.15 g. of material from the first ammonium oxalate extract was refluxed for 2 hr. in 15 ml. of 1% sulfuric acid. The hydrolyzate was neutralized with barium carbonate and then filtered. A chromatogram (Figure 11) of the hydrolyzate and known L-rhamnose, D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose, and D-galacturonic acid was prepared. The chromatogram was developed with
Figure 11. Chromatogram of a Hydrolyzate of the Ammonium Oxalate Extract
40/19/11 :: 1-butanol/water/ethanol for ca. 12 hr. Galactose, mannose, arabinose, rhamnose (or apiose) (69), and two, slow-moving inconclusively identified spots, one of which was probably galacturonic acid, were found.

Mannose and arabinose have nearly identical $R_f$ values in this developer. The identification was based on the color of the hydrolyzate spot (orange-red) opposite known mannose (yellow-brown) and known arabinose (pink-red).

Further confirmation of mannose and arabinose was obtained by electrochromatography, by which method mannose and arabinose are readily separated.

6. Holocellulose Preparation

An amount of 336.0 g. (dry wt.) of residue from the second ammonium oxalate extraction was stirred into 10 liters of distilled water in a 3-gal. bottle at 75 $^\circ$C. A steady stream of nitrogen was bubbled into the mixture. Amounts of 137.5 g. of sodium chlorite were added every 22-23 min. for 1.5 hr. (550.0 g., total of sodium chlorite). At the first addition of sodium chlorite, ca. 50 ml. of glacial acetic acid was added to adjust the mixture to pH 4.5-5.0. At the subsequent additions of sodium chlorite, a few more milliliters of glacial acetic acid were added, making a total of 80 ml. used during the reaction. An amount of 325 ml. of isoamyl alcohol was
added, mainly during the first half of the reaction, to prevent foaming. This is a modification of the procedure of Whistler and co-workers (52).

The hot reaction mixture was rapidly filtered through milk filter-disks (68) in a large Bürchner funnel and washed with 20 liters of distilled water and 1 liter of acetone. The filtrate and wash liquids were drawn directly into the aspirator and flushed down the drain. The residue (292.0 g., dry wt.) was a white powder (ash, 0.91% and 0.94% as sulfate). This residue, coffee "holocellulose", amounted to 40.6% of the dry weight of the original bean. The holocellulose gave a negative ferric chloride test, a negative ninhydrin test, and a positive Molisch test.

7. Extraction of Coffee Holocellulose with Alkali

Samples of coffee holocellulose were extracted with aqueous solutions of 5%, 10%, and 20% (by weight) aq. potassium hydroxide. Amounts of 25.0 g. (dry wt.) of holocellulose were stirred in 250 g. of alkali for 24 hr. at 25 ±2° under nitrogen.

The resulting viscous, slightly yellow mixtures were filtered through a Bürchner-type funnel with fritted-glass disc (med. porosity). The residues were washed with 250 g. of potassium hydroxide (5%, 10%, or 15%) and 200 ml. of water. The filtrates and wash liquids were neutralized with 50% acetic acid in an ice bath. Centrifugation of the neutralized filtrates yielded the hemicellulose-A fraction (a coarse, dense, grey powder). To each of the decantates from the centrifugation were added 3 volumes of abs. ethanol to precipitate the hemicellulose-B fraction (a fine, light, cream-colored powder). The
hemicellulose fractions were dried by successive washing and centrifugation with abs. ethanol and ether. Final drying was accomplished in a vacuum desiccator over phosphorus pentoxide.

The alkali-insoluble residues were further washed with 50 ml. of 10% acetic acid, 200 ml. of water, and several portions of abs. ethanol. The material was dried in air and then in a vacuum desiccator over phosphorus pentoxide.

The results of several extractions are compared in Table 1.

a. Hydrolysis of the Alkali Fractions with Sulfuric Acid

An amount of 25 mg. of hemicellulose-A was refluxed for 2.5 hr. in 2.5 ml. of N sulfuric acid (hydrolysis not complete). The dark-brown solution was neutralized with barium carbonate and filtered. The filtrate gave no sugar spots when chromatographed on paper.

An amount of 0.15 g. of hemicellulose-B was refluxed for 1.5 hr. in 15 ml. of 1% sulfuric acid (hydrolysis not complete). Another amount of 0.15 g. of hemicellulose-B was refluxed for 3 hr. in 15 ml. of N sulfuric acid (hydrolysis not complete). Also an amount of 0.15 g. of alkali-insoluble residue was refluxed for 23.5 hr. in N sulfuric acid (no insoluble residue but hydrolysis probably not complete). The resulting yellow hydrolyzates were neutralized with barium carbonate and filtered.

A chromatogram of the neutral hydrolyzates of the alkali-insoluble holocellulose and hemicellulose-B (both 1% and N sulfuric acid) was prepared. Known D-glucose, D-xylose, L-arabinose, L-rhamnose, D-mannose, D-galactose, and D-galacturonic acid were applied
<table>
<thead>
<tr>
<th>Concentration of Potassium Hydroxide (by weight)</th>
<th>Weights of Alkali Fractions from 25-g. Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alkali-Insoluble</td>
</tr>
<tr>
<td>5%</td>
<td>21.7 g.</td>
</tr>
<tr>
<td>10%</td>
<td>20.3 g.</td>
</tr>
<tr>
<td>20%</td>
<td>21.5 g.</td>
</tr>
</tbody>
</table>

Table 1. Extraction of Coffee Holocellulose with Aqueous Potassium Hydroxide
to the paper. The chromatogram (Figure 12) was developed for 40 hr. with 40/19/11 : : 1-butanol/water/ethanol. The sugar spots were located by spraying with 2% p-anisidine hydrochloride in 1-butanol.

Rhamnose (or apiose) (69), xylose, arabinose, galactose, a trace of mannose, and several slow-moving inconclusively identified spots (probably partial hydrolysis products) were detected in the hydrolyzates of hemicellulose-B.

Galactose, mannose, and one unidentified, slow-moving spot were detected in the hydrolyzate of the alkali-insoluble residue.

An electrochromatogram of the neutral hydrolyzate of the alkali-insoluble holocellulose (Figure 13) and known L-arabinose, D-glucose, D-galactose, and D-mannose was prepared. The sugars were located by spraying with aniline phthalate reagent (1.66 g. phthalic acid and 0.93 g. aniline dissolved in 100 ml. of water-saturated 1-butanol).

Mannose, galactose, arabinose, and glucose were detected. The glucose spot was weak.

Another chromatogram of the neutral hydrolyzate of the alkali-insoluble residue was prepared with known D-glucose, D-galactose, and D-mannose. This chromatogram (Figure 14) was developed 60 hr. with 10/3/3 : : 1-butanol/pyridine/acetic acid and the sugar spots were detected with 2% p-anisidine in 1-butanol.

The sugar spots detected were galactose, mannose, glucose, arabinose (by pink-orange color of mannose spot) and a slow-moving spot (probably galacturonic acid).
Hydrol. of Hemicellulose-B

1.5 hr.
1% 
H₂SO₄

3 hr.
N H₂SO₄

Hydrol. of Alkali-Insol. Residue

D-Galacturonic acid

D-Glucose

D-Galactose

L-Arabinose

D-Mannose

D-Xylose

L-Rhamnose

Figure 12. Chromatogram of Some Holocellulose Hydrolyzates
Figure 13. Electrochromatogram of a Hydrolyzate of Alkali-Insoluble Coffee Holocellulose
(YB = yellow-brown; PR = pink-red; Or = orange; Y = yellow)
Figure 14.
Chromatogram of a Hydrolyzate of Alkali-Insoluble Holocellulose
(B = brown, Y = yellow, Or = orange)

Chemicals:
- D-Glucose
- D-Galactose
- D-Mannose
- Hydrolyzate of Alkali-Insoluble Holocellulose
- D-Galactose
- D-Glucose
- D-Mannose
The hydrolyzate of the alkali-insoluble residue gave a negative Seliwanoff test for ketoses.

b. Hydrolysis of Alkali-Insoluble Holocellulose with 3% Nitric Acid

An amount of 1.000 g. of 10% potassium hydroxide-insoluble holocellulose was refluxed 72 hr. in 3% nitric acid. The unhydrolyzed residue (40 mg.) was recovered and subsequently hydrolyzed 1.5 hr. in 72% sulfuric acid (10 drops), diluted with 20 volumes of water, and heated in a boiling water bath for 1 hr. The solution was neutralized with barium carbonate and filtered.

The filtrate and known D-glucose, D-galactose, D-mannose, D-glucuronic acid, D-galacturonic acid, and D-glucuronolactone were chromatographed 12 hr. with 40/19/11 : 1-butanol/water/ethanol and sprayed with p-anisidine reagent (Figure 15). Glucose and an inconclusively identified slow-moving spot (brown color, probably a uronic acid) were detected in the hydrolyzate.

8. Lignin Determination

The amounts of lignin in several of the coffee fractions were determined by the method of Ritter and Barbour (70). A sample (1-2 g.) of the material to be tested was placed in a weighed 50-ml. round-bottomed flask and 25 ml. of 72% sulfuric acid were added. After standing 2 hr. at 20° with occasional shaking a clear, almost colorless solution was obtained. This solution was quantitatively washed into 600 ml. of distilled water in a 1-liter round-bottomed
Figure 15. Chromatogram of a Hydrolyzate of the Insoluble Residue from the Partial Hydrolysis of Alkali-Insoluble Holocellulose with 3% Nitric Acid
flask and refluxed for 4 hr. The final hydrolyzates were nearly colorless.

The hydrolyzates were filtered through sintered-glass filter crucibles, washed, dried 1 hr. at 100°, and weighed.

The lignin values for some of the coffee fractions are given in Table 2.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original Bean</td>
<td>2.15 and 2.65</td>
</tr>
<tr>
<td>Holocellulose (40.6% of Original Bean)</td>
<td>1.77 and 1.82</td>
</tr>
<tr>
<td>10% KOH-insol. Holocellulose (35.9% of Original Bean)</td>
<td>0.3 and 0.4</td>
</tr>
</tbody>
</table>

A sample of lignin from the holocellulose was analyzed for carbon, hydrogen, methoxyl, and ash content. The values obtained were: C, 59.22; H, 7.99; MeO, 3.07, and Ash, 3.64.

E. Further Characterization of the Alkali-Insoluble Coffee Holocellulose

1. Extraction with Cuprammonium Solution

An amount of 10.0 g. of alkali-insoluble holocellulose was stirred into 250 ml. of cuprammonium solution (71) in a 500-ml.

(71) H. F. Launer and W. K. Wilson, Anal. Chem., 22, 455 (1950). An amount of 15.9 g. of cuprous hydroxide was dissolved in 1 liter of 28% ammonia by stirring strongly in an ice bath for 30 min.
round-bottomed flask. The flask was tightly stoppered and kept 8 hr. at 10-20° and 20 hr. at 25-30°. The mixture was filtered through a sintered-glass funnel. The residue (neutralized with 18 N sulfuric acid in an ice bath) was washed with water until only a pale green tint remained, then dried at room temperature; yield, 7.0 g. The filtrate (neutralized with 18 N sulfuric acid in an ice bath) yielded a white flocculent precipitate which was collected by filtration and dried in air at room temperature; yield, 1.1 g.

An amount of ca. 15 mg. of cuprammonium-soluble material was refluxed 24 hr. in 1 N sulfuric acid (hydrolysis possibly not complete but no insoluble residue). The solution was neutralized with barium carbonate and filtered. The neutralized hydrolysate was electrochromatographed and sprayed with aniline phthalate reagent. A strong mannose spot, a glucose spot, and a weak galactose spot were detected (Figure 16).

2. Partial Hydrolysis

An amount of 1.000 g. of 10% alkali-insoluble holocellulose was placed in a 100-ml. round-bottomed flask. A volume of 50 ml. of boiling 1.5% sulfuric acid was added and the mixture was refluxed 5 min. The mixture was rapidly filtered through a fritted-glass funnel; the filtrate was neutralized with barium carbonate, filtered, and stored at -25°.

The unhydrolyzed residue was returned to the reflux flask with a fresh portion of boiling 1.5% sulfuric acid. The hydrolysis procedure was repeated a number of times gradually increasing the contact time.
Figure 16. Paper Chromatogram of D-Galactose, Hydrolzate of Cuprammonium-Soluble Holocellulose, and D-Glucose.
The neutralized hydrolysates and known \( \text{D-galactose, D-mannose,} \) and \( \text{L-arabinose} \) were chromatographed on paper using \( 40/19/11 : 1\)-butanol/water/ethanol as the developer and \( 2\% \) \( p\)-anisidine hydrochloride in \( 1\)-butanol as the spray reagent. The intensities of the various sugar spots given by the hydrolysates and the duration of hydrolysis are given in Table 3.

### Table 3. Selective Hydrolysis of Alkali-Insoluble Holocellulose

<table>
<thead>
<tr>
<th>Contact Time (Min.)</th>
<th>Spot Intensity (Est. Visually)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cumulative Interval Arabinose</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>45</td>
<td>15</td>
</tr>
<tr>
<td>60</td>
<td>15</td>
</tr>
<tr>
<td>90</td>
<td>30</td>
</tr>
<tr>
<td>120</td>
<td>30</td>
</tr>
<tr>
<td>180</td>
<td>60</td>
</tr>
<tr>
<td>240</td>
<td>60</td>
</tr>
<tr>
<td>300</td>
<td>60</td>
</tr>
<tr>
<td>360</td>
<td>60</td>
</tr>
</tbody>
</table>

a A non-migrating spot (yellow color with \( p\)-anisidine reagent) was present. Later evidence (see Section 2. C. below) indicated the presence of galactose in a mild hydrolyzate (15 min. in 1.5\% sulfuric acid).

b The mannose and arabinose spots overlap making it difficult to estimate the amount of mannose in the presence of significant amounts of arabinose.

The insoluble residue from the 6-hr. hydrolysis with 1.5\% sulfuric acid was dissolved in 25 ml. of 72\% sulfuric acid, let stand 2 hr. at room temperature, diluted to 600 ml. and refluxed 4 hr. The hydrolyzate was neutralized with barium carbonate, filtered, and lyophilized.
Electrochromatography of the lyophilized solids with known D-mannose, D-galactose, and D-glucose indicated the presence of mannose and glucose only (Figure 17).

3. Estimation of the Sugar Ratio in the Alkali-Insoluble Coffee Holocellulose

a. Anhydroarabinose

The method of Bates et al. (72) was employed for the determination of pentoses.

An amount of 0.070 g. of arabinose was placed in a 250-ml. round-bottomed flask and 100 ml. of 12% hydrochloric acid was added. The flask was attached to a distilling head and condenser. The rate of heating of the flask was adjusted to produce ca. 30 ml. of distillate in 10 min. The distillate was collected in 30-ml. amounts and the flask was refilled until 360 ml. of distillate had been collected. An amount of 17 ml. of phloroglucinol solution (11 g./1800 ml. of 12% hydrochloric acid) was added and 12% hydrochloric acid was added to make the volume up to 400 ml. After standing overnight at room temperature, the black precipitate was filtered and washed with 150 ml. of water. The precipitate was dried 1 hr. at 100° and weighed; yield, 0.055 g. From Kröber's tables (73), the weight of arabinose


Figure 17. Electrophoresis of the Insoluble Residue from the 1.5% Sulfuric Acid Hydrolysis of the Alkali-Insoluble Holocellulose

- D-Manose
- Hydrolyzate of the Insoluble Residue from the 1.5% Sulfuric Acid Hydrolysis of Alkali-Insoluble Holocellulose
- D-Galactose
- D-Glucose
corresponding to the precipitate formed is 0.067 g.

The determination of anhydropentose in the alkali-insoluble holocellulose was carried out in the same manner using 1,000-g. samples. The weights of precipitate obtained and the corresponding weights of anhydroarabinose from Kröber's tables are given in Table 4.

Table 4. Estimation of Arabinose as the Phloroglucide

<table>
<thead>
<tr>
<th>Sample</th>
<th>Wt. of Phloroglucide</th>
<th>Wt. of Anhydroarabinose (Kröber)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.070 g. arabinose</td>
<td>0.055</td>
<td>0.067</td>
</tr>
<tr>
<td>1,000 g. alkali-insol. coffee holocellulose</td>
<td>0.064</td>
<td>0.067</td>
</tr>
<tr>
<td>1,000 g. alkali-insol. coffee holocellulose</td>
<td>0.060</td>
<td>0.063</td>
</tr>
<tr>
<td>1,000 g. alkali-insol. coffee holocellulose</td>
<td>0.067</td>
<td>0.070</td>
</tr>
</tbody>
</table>

The average of the three values corresponds to 6.7% anhydroarabinose in the alkali-insoluble coffee holocellulose.

b. Anhydrogalactose

The procedure of Hirst, Jones, and Woods (74) for the estimation of galactose as the 1-methyl-1-phenylhydrazone was followed.

Mannose and arabinose both interfere with the estimation of galactose by this method. From an earlier experiment on the hydrolysis of the alkali-insoluble holocellulose with 1.5% sulfuric acid it was
concluded that the arabinose could be readily removed by a 15-min. hydrolysis with boiling 1.5% sulfuric acid leaving galactose and mannose in the unhydrolyzed residue. (It was later found that some of the galactose is removed with the arabinose.) This residue could then be hydrolyzed by 72% sulfuric acid and the mannose fermented by a galactose-negative yeast.

Two samples (I, 0.993 g. and II, 1.170 g.) of alkali-insoluble coffee holocellulose were each refluxed 15 min. with 50 ml. of 1.5% sulfuric acid. The solutions were then quickly filtered through fritted-glass funnels. The filtrates were neutralized with barium carbonate, filtered, and lyophilized.

The residues from the mild hydrolysis were lyophilized. The dry residues were each placed in 25 ml. of 72% sulfuric acid in 50-ml. round-bottomed flasks and kept at 20° for 2 hr. The solutions were each washed into 575 ml. of water in 1-liter flasks and refluxed 4 hr. The hydrolyzates (colorless) were filtered and the dried residues (light brown, pos. Molisch) were weighed as lignin; yield, (I) 0.023 g. and (II) 0.051 g.

The filtrates were neutralized with barium carbonate, filtered, and concentrated to ca. 25 ml. at 50-60° on a hot water bath. The concentrates were transferred to 50-ml. volumetric flasks and adjusted to the mark with water. Amounts of 25 ml. of each were transferred by pipet to 250-ml. round-bottomed flasks and designated Iₐ and IIₐ. The remaining 25 ml. of each sample were rinsed into 250-ml. flasks and designated Iₐ and IIₐ. All four of the solutions were lyophilized.
Samples I \(a\) and II \(a\) were dissolved in 25 ml. of water and 5.0 ml. of a thick slurry (ca. 0.5% g., dry wt.) of freshly grown yeast (75).

(75) No. 17052, galactose-negative, from a tube-slant kindly supplied by Dr. Carl C. Lindgren, Director, Biological Research Laboratory, Southern Illinois University, Carbondale, Illinois.

were added. The evolution of gas (carbon dioxide), indicating the start of fermentation, was noted within 10 min. After 3 hr. at 27-28°, electrochromatography revealed only a very small amount of mannose. After 6 hr. at 27-28° no trace of mannose could be found on electrochromatography. The yeast was removed by filtration and the filtrates were lyophilized. Control samples, each containing 300 mg. of D-mannose and various amounts of D-galactose were processed in the same manner (see Table 5). (The yeast was grown in the following sterilized medium: 7.0 g. of Difco Yeast Extract (76),

(76) Manufactured by Difco Laboratories, Inc., Detroit, Mich.

5.0 g. of potassium dihydrogen phosphate, 100.0 g. of glucose, and 1000 ml. of water.)

Samples I \(a\) and II \(a\) were each transferred to 50-ml. round-bottomed flasks with 10 ml. of water. An amount of 10 ml. of a mixture of 1-methyl-1-phenylhydrazine (25 ml.) and abs. ethanol (100 ml.) containing 3 ml. of glacial acetic acid was added to each flask. The tightly stoppered flasks were kept at 32° for 12 hr. and at 0° for 9 hr. No crystals or precipitate formed. Sample I \(a\) was seeded with known galactose 1-methyl-1-phenylhydrazone but no
other crystals formed. An amount of 150 mg. of known galactose was added to Sample II_. The sample was kept at 32° for 12 hr. and 0° for 9 hr. The crystals of galactose 1-methyl-1-phenylhydrazone were collected in a sintered-glass filter crucible, washed with 10 ml. of ice-cold ethanol, dried 30 min. at 100° and weighed; yield 203 mg. of white crystals. The yield in this case was well within the experimental error of the average of a number of other determinations (Table 5). The lyophilized 1.5% sulfuric acid-hydrolyzates of

Table 5. Estimation of Galactose as the 1-Methyl-1-phenylhydrazone

<table>
<thead>
<tr>
<th>Wt. of Galactose</th>
<th>Wt. of Hydrazine</th>
<th>Wt. of Theoretical Yield</th>
<th>% of Theoretical Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>220 mg.</td>
<td>292 mg.</td>
<td>347 mg.</td>
<td>86.2</td>
</tr>
<tr>
<td>200 mg.</td>
<td>273 mg.</td>
<td>316 mg.</td>
<td>86.4</td>
</tr>
<tr>
<td>200 mg.</td>
<td>269 mg.</td>
<td>316 mg.</td>
<td>85.2</td>
</tr>
<tr>
<td>200 mg.</td>
<td>264 mg.</td>
<td>316 mg.</td>
<td>83.6</td>
</tr>
<tr>
<td>160 mg.</td>
<td>210 mg.</td>
<td>252 mg.</td>
<td>83.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Avg.</td>
<td>84.6 ±2</td>
</tr>
</tbody>
</table>

150 mg. (added to Sample I) 203 mg. 237 mg. 85.7

Samples I and II were each dissolved in a few milliliters of water filtered (to remove barium sulfate) and washed quantitatively into 50-ml. round-bottomed flasks. The volume of solution in each flask was adjusted to 10 ml. An amount of 10 ml. of a mixture of 1-methyl-1-phenylhydrazine (25 ml.) and abs. ethanol (100 ml.) containing 3 ml. of glacial acetic acid was added to each flask. The tightly stoppered flasks were kept at 32° for 12 hr. and at 0° for 9 hr. No precipitate formed even after seeding with D-galactose 1-methyl-1-phenylhydrazone.
c. Anhydromannose

Samples I₈ and II₈ were transferred to 50-ml. round-bottomed flasks with 10 ml. of water. An amount of 10 ml. of a mixture of phenylhydrazine (25 ml.) and abs. ethanol (100 ml.) containing 3 ml. of glacial acetic acid was added to each flask. The tightly stoppered flasks were kept at 32° for 15 hr. and 0° for 12 hr. The phenylhydrazone was collected in sintered-glass filter crucibles, washed with 10-15 ml. of ice-cold ethanol, dried 30 min. at 100° and weighed; yield I₈, 0.373 g. and II₈, 0.452 g. of almost colorless crystals. The corresponding amounts of anhydromannose would be 0.224 g. and 0.271 g. or 45.2% and 46.3%, respectively (minimum values).

4. Some Derivatives of the Constituent Sugars

a. Selective Hydrolysis

An amount of 10.00 g. of 10% potassium hydroxide insoluble coffee holocellulose was added to 250 ml. of boiling 1.5% sulfuric acid and refluxed 15 min. The mixture was filtered; the filtrate was neutralized with barium carbonate, filtered, and lyophilized. After drying over phosphorus pentoxide 2.0 g. of white solids, which tended to sirup, were obtained.

Paper chromatography of the 1.5% hydrolyzate with known L-arabinose, D-galactose, D-glucose, and D-mannose indicated the presence of arabinose and galactose (Figure 18).

The unhydrolyzed residue (white solid) was also lyophilized. The dry material was added to 250 ml. of cold 72% sulfuric acid in a 500-ml. round-bottomed flask and shaken vigorously for 2 hr. until all of the material had dissolved. The solution (very slightly
Figure 18. Chromatogram of a Partial Hydrolyzate of Alkali-insoluble Holocellulose with 1.5\% Sulfuric Acid
(B = brown, P = pink)
colored) was poured into 4 liters of water in a 5-liter round-bottomed flask and refluxed for 4 hr. The solution was neutralized with barium carbonate, filtered, concentrated (67) to ca. 300 ml. and lyophilized. An amount of ca. 7.5 g. of thick brown sirup was obtained.

b. Acetylation of the 72% Sulfuric Acid Hydrolysate and Chromatography of the Acetylated Product

An amount of 5.2 g. of the brown sirupy 72% sulfuric acid hydrolysate, 2.5 g. of anhydrous sodium acetate, and 30 ml. of acetic anhydride were placed in a 500-ml. round-bottomed flask and heated to incipient boiling for 2-3 min. while swirling over a bunsen flame. The brown solution was poured into 400 ml. of ice water and left overnight. The following day the mixture was extracted with chloroform. The chloroform extract was washed, dried, and concentrated to a thick brown sirup; yield 8.9 g. On standing at -25°, 0.6 g. of crude crystals formed and were removed by filtration; yield, after re-crystallization from ethanol, 230 mg. of fine white needles, m.p. 128-130°.

An amount of 1.0 g. of acetate sirup in 100 ml. of benzene was added at the top of a column (250 x 75 mm., diam.) of 5 parts of Magnesol (77) to 1 part of Celite No. 535 (58) (wt. ratio) prewet

(77) A hydrated magnesium acid silicate produced by the Westvaco Chemical Division of the Food Machinery and Chemical Corp., South Charleston, West Virginia.

with 150 ml. of benzene. The column was developed with 2500 ml. of 200/1 benzene/t-butyl alcohol (vol. ratio). Two zones
(poorly resolved) and some tightly adsorbed substances at the top of the column (Figure 19) were located by streaking the extruded column with permanganate indicator (1% potassium permanganate in 10% sodium hydroxide). The two zones were numbered from the top to the bottom and sectioned. The sugar acetate in each was removed by extraction with acetone and the acetone solutions evaporated to sirups. Four such columns were prepared.

The material from Zone 2 (ca. 1.95 g., decolorized with carbon) crystallized from ethanol in the cold; yield 0.16 g., m.p. 129-130°, mixed m.p. 130-132° on admixture with an authentic specimen, $[\alpha]_{D}^{20} +6.8^\circ$ (c 3.4, chloroform). This identifies the substance as penta-O-acetyl-$\beta$-D-glucopyranose; accepted constants, m.p. 135, $[\alpha]_{D}^{20} +3.8^\circ$.

The material from Zone 1 (ca. 0.7 g., decolorized with carbon) crystallized from ethanol; yield 0.41 g., m.p. 112-113°, mixed m.p. 113-115° on admixture with an authentic specimen, $[\alpha]_{D}^{20} -20.7^\circ$ (c 3.3, chloroform). This identifies the substance as penta-O-acetyl-$\beta$-D-mannopyranose; accepted constants, m.p. 117-118°, $[\alpha]_{D}^{20} -25.2^\circ$.

Electrochromatography of portions of the de-acetylated mother liquors indicated that mannose was the main constituent of each. Glucose was also present in both mother liquors.

c. Acetylation of the 1.5%-Sulfuric Acid Hydrolyzate and Chromatography of Acetylated Product

An amount of 1.0 g. of the 1.5%-sulfuric acid hydrolyzate, 0.5 g. of anhydrous sodium acetate; and 5 ml. of acetic anhydride were placed in a 250-ml. round-bottomed flask and heated to incipient
Figure 19. Chromatogram of the Acetylated 72%-Sulfuric Acid Hydrolyzate on Magnesol-Celite
boiling for 2-3 min. while swirling over a bunsen flame. The brown solution was poured into 200 ml. of ice water and left overnight. The following day the mixture was extracted with chloroform. The chloroform extract was washed, dried, and concentrated to a thick brown sirup; yield 1.9 g.

An amount of 1.0 g. of this material in 100 ml. of benzene was added at the top of a column (250 x 75 mm., diam.) of 5 parts of Magnesol to 1 part of Celite No. 535 (wt. ratio), prewet with 100 ml. of benzene, and developed with 1000 ml. of 500/1 : benzene/t-butyl alcohol (vol. ratio). Only one zone was detected by streaking the extruded column with permanganate indicator (Figure 20). The sugar acetate was removed by extraction acetone and the acetone solution evaporated to a sirup. Two such columns were prepared.

The material from the zone (decolorized with carbon) failed to crystallize from ethanol or from a sirup. Electrochromatography of the de-acetylated material indicated the presence of arabinose and lesser amounts of galactose and mannose.

d. Characterization of Mannose Phenylhydrazone from Coffee

An amount of ca. 1 g. of 72% sulfuric acid hydrolyzate was treated by the procedure used in the estimation of mannose (see Section III, E. 1. b.); yield, 0.415 g. of almost white crystals, m.p. 166-188°, mixed m.p. 186-188° on admixture with an authentic specimen, $[\alpha]_D^{22} +23.3^\circ$ (c 0.1, pyridine).
Figure 20. Chromatogram of the Acetylated 1.5% Sulfuric Acid Hydrolyzate on Magnesol-Celite
5. Acetylation of Alkali-Insoluble Coffee Holocellulose and Fractionation of the Acetylated Product

a. Pyridine-Acetic Anhydride Acetylation

A modification of the method of Carson and Maclay (78) was first used to acetylate the alkali-insoluble holocellulose.

(1) First Acetylation

An amount of 10.00 g. of dry 10% potassium hydroxide-insoluble holocellulose was stirred overnight in 250 g. of formamide (re-distilled) at 65 °C. The material swelled considerably but did not dissolve noticeably. An amount of 250 ml. of pyridine (distilled over barium oxide) was added and the mixture was cooled to ca. 25°. With continued stirring, ca. 100 ml. of acetic anhydride was added in five portions over a six-hour period. The stirring was continued 18 hr. at room temperature.

The reaction mixture was filtered (fritted-glass funnel) and the filtrate was poured into 3 liters of water containing 1 kg. of ice. After standing 24 hr. a black precipitate (50 mg.) formed and was recovered by filtration. The insoluble residue from the reaction mixture was dark brown, granular, and partly gelatinized. This residue was poured into 2 liters of ice water and let stand 15 min. The insoluble, yellow-brown residue was recovered by filtration. The residue was washed with alcohol, dried 24 hr. in air at room temperature and 12 hr. at 70° in a vacuum oven; yield, 13.83 g. of granular grey-cream solids.
(a) Acetyl Estimation

The amount of acetyl in the insoluble solids was determined by distillation with p-toluenesulfonic acid in the apparatus shown in Figure 21.

A 0.2-0.65 g. sample (accurately weighed) of acetylated material was placed in the 100-ml. flask (A) containing ca. 50 ml. of 40\% p-toluenesulfonic acid. The flask was heated with a small ceramic-type electric heater (B) adjusted (variable autotransformer) to give ca. 100 ml. of distillate per hour. The 300-ml. constant-head dropping funnel (C) was adjusted to maintain a volume of 40-60 ml. in the distilling flask. The distillate was collected in the receiver (D). Before the addition of the sample a control distillate of ca. 300 ml. was collected and titrated with 0.1004 N sodium hydroxide using phenolphthalein as an indicator. The usual "blank" obtained was 0.05 ml. of 0.1004 N sodium hydroxide per 100 ml. of distillate. After the addition of the sample, 300-ml. amounts of distillate were collected and titrated until the amount of 0.1004 N sodium hydroxide required was again 0.05 ml. per 100 ml. of distillate or reached a constant value. The total amount of distillate required for a sample varied from 1500 ml. to 2800 ml. and depended mainly upon the ease with which the sample was wet by the p-toluenesulfonic acid solution.

Before removing the receiver for titration of the distillate, the heater was turned off and water was circulated through the reflux condenser (E) to prevent the loss of any acetic acid.
Figure 21. Apparatus for Acetyl Estimation
Amounts of 37.23 ml. and 19.11 ml. of 0.100 N sodium hydroxide were required to neutralize the acetic acid produced by distilling 0.4165 g. and 0.2167 g., respectively, of cellulose acetate (79) by distilling with p-toluenesulfonic acid (2100 ml. and 1450 ml. of distillate, respectively). These results indicate acetyl values of 38.7% and 38.0%, respectively. An amount of 21.25 ml. of 0.1004 N sodium hydroxide was required to neutralize the acetic acid produced by distilling 0.2767 g. of insoluble residue from the first pyridine-acetic anhydride acetylation with p-toluenesulfonic acid (2700 ml. of distillate). The material thus contained 32.9% acetyl.

(2) Second Acetylation

An amount of 10.00 g. of the partially acetylated solids from the first acetylation was stirred 22 hr. at 65 °C in 250 g. of formamide. About one-fourth of the material was noticeably swollen. A volume of 250 ml. of pyridine was added and the mixture was cooled to 43°. A volume of 100 ml. of acetic anhydride was added and the temperature of the mixture rose to 45°. The mixture was stirred 2 hr. and then let stand 48 hr. at room temperature.

The black reaction mixture was filtered, washed with pyridine, and the filtrate poured into 3 liters of water containing 1 kg. of ice. On standing overnight, a dark-grey gelatinous precipitate formed. This material was recovered by decantation and centrifugation.
and purified by pouring a chloroform solution of it into methanol; yield, 0.265 g. of grey-white, friable solids (after drying in air).

The insoluble material obtained by filtration of the reaction mixture was placed in 1 liter of water containing 1 kg. of ice and after a few minutes was recovered by filtration. The granular, tan residue was washed with water and alcohol, dried in air, and finally for 12 hr. at 70° in a vacuum oven; yield, 10.21 g. of light tan solids.

An amount of 53.68 ml. of 0.1004 N sodium hydroxide was required to neutralize the acetic acid produced by distilling 0.6409 g. of insoluble residue from the second pyridine-acetic anhydride acetylation with p-toluenesulfonic acid (2800 ml. of distillate). The material thus contained 36.2% acetyl.

(3) Third Acetylation

An amount of 7.76 g. of insoluble product from the second acetylation (dried 2 hr. at 96° in a vacuum oven) was placed in a dry 500-ml. two-necked round-bottomed flask fitted with a stirrer and potassium hydroxide-filled drying tube. A volume of 250 ml. of pyridine (freshly distilled over barium oxide) was added and the flask was placed on a hot water bath at 96-99°. The mixture was strongly stirred and 25 ml. of acetic anhydride was added at the start and every 15 min. over a 45-min. period (total of 100 ml. of acetic anhydride). After 2 hr., 50 ml. more acetic anhydride was added. The mixture was heated another 2 hr. and then allowed to stand overnight at room temperature. The reaction mixture was
treated as before. From the filtrate was obtained 0.800 g. of grey friable solids (after precipitation from methanol and drying in air). The insoluble reaction product was a granular orange-brown material; yield, 7.36 g.

An amount of 19.91 ml. of 0.100 M sodium hydroxide was required to neutralize the acetic acid produced by distilling 0.2382 g. of insoluble residue from the third pyridine-acetic anhydride acetylation with p-toluenesulfonic acid (2000 ml. of distillate). The material thus contained 36.2% acetyl.

An amount of ca. 50 mg. of soluble product from the second acetylation reaction was dissolved in ca. 5 ml. of chloroform and layered over 10 ml. of 45% KOH in a 25-ml. round-bottomed flask. The flask was placed in a hot water bath and shaken occasionally. As the chloroform evaporated it was replaced several times by fresh chloroform and eventually by acetone. After 2 hr. the de-acetylated material appeared as a white precipitate in the aqueous layer and was recovered by filtration. The residue was hydrolyzed 1 hr. at 20° with 72% sulfuric acid, diluted to approximately 3% sulfuric acid and refluxed 3 hr. The solution was neutralized with barium carbonate, filtered, and chromatographed. Only mannose was detected by paper chromatography. Mannose and a trace of galactose were detected by electrochromatography.

b. Trifluoroacetic Anhydride-Acetic Acid Acetylation

A modification of the method of Barclay, Bourne, Stacey, and
Webb (80) for the acetylation of a bacterial cellulose was employed.


An amount of 5.00 g. of 10% potassium hydroxide-insoluble holo-cellulose (previously kept over phosphorus pentoxide) was added quickly to a well-stirred mixture of 122 ml. of trifluoracetic anhydride and 77 ml. of acetic acid in a 500-ml. two-necked, round-bottomed flask in an ice bath. The mixture, protected from atmospheric moisture with a potassium hydroxide-filled drying tube, was stirred 4 hr. (125 ml. of chloroform added after 3 hr.) in the ice bath and 1 hr. after removal from the ice bath.

The reaction mixture (turbid and quite viscous) was poured into 1 liter of petroleum ether (b.p. 30-60°). A white, curdy precipitate formed. The petroleum ether solution was decanted into aqueous sodium bicarbonate and left overnight in the hood. The white precipitate was extracted with three ca. 300-ml. portions of chloroform by boiling in a large beaker. The combined chloroform extracts were poured into another portion of aqueous sodium bicarbonate.

About 1 liter of chloroform was added to the petroleum ether solution and it was separated from the aqueous layer in a large separatory funnel. The extract was washed, dried, and concentrated to sirup. The sirup was transferred to a weighed round-bottomed flask and dried at 50° for 18 hr. in a vacuum oven; yield, 1.70 g. of brittle, slightly yellow film. This material was designated Fraction PA-1.
An amount of 19.52 ml. of 0.1004 N sodium hydroxide was required to neutralize the acetic acid produced by distilling 0.2009 g. of Fraction PA-1 with p-toluenesulfonic acid. The material thus contained 42.0% acetyl.

**Anal.** The calculation of carbon and hydrogen values for an acetylated polysaccharide composed of anhydropentose and anhydrohexose units of unknown ratio is a complicated matter. The amount of anhydropentose in the alkali-insoluble holocellulose was found to be 6.7%. In the acetylated fractions, all of the arabinose was found in PA-1 and PA-2 which constituted one-half of the weight of the acetylated material. Therefore, it was assumed that the original polysaccharides of PA-1 and PA-2 were each ca. 14% anhydropentose and 86% anhydrohexose and that the amount of hydrogen on non-acetylated hydroxyls was negligible. The error involved in the assumption of a value of ca. 14% for the anhydropentose is negligible since the carbon-hydrogen-oxygen ratios in C\textsubscript{5}H\textsubscript{10}O\textsubscript{4} and C\textsubscript{6}H\textsubscript{7}O\textsubscript{5} are so similar.

Calcd. for 42.0% C\textsubscript{2}H\textsubscript{3}O, 8.12% C\textsubscript{5}H\textsubscript{6}O\textsubscript{4}, and 49.88% C\textsubscript{6}H\textsubscript{7}O\textsubscript{5}: C, 49.74; H, 5.63. Found: C, 50.59; H, 5.96 (corr. for 0.54% ash, not alkaline). Re-calcd. for 44.08% C\textsubscript{2}H\textsubscript{3}O (maximum possible), 7.83% C\textsubscript{5}H\textsubscript{6}O\textsubscript{4}, and 48.09% C\textsubscript{6}H\textsubscript{7}O\textsubscript{5}: C, 50.01; H, 5.61.

The combined chloroform extracts of the material precipitated by the addition of the reaction mixture to petroleum ether were washed, dried, concentrated to a sirup, and finally dried at 50° for 18 hr. in a vacuum oven; yield, 2.50 g. of pliable, slightly yellow film. This material was designated Fraction PA-2.
An amount of 17.00 ml. of 0.1004 N sodium hydroxide was required to neutralize the acetic acid produced by distilling 0.1879 g. of Fraction PA-2 with p-toluenesulfonic acid. The material thus contained 39.2% acetyl.

Anal. Calcd. for 39.2% C₂H₃O, 8.51% C₅H₆O₄, and 52.29% C₆H₇O₅: C, 49.49; H, 5.47. Found: C, 49.63; H, 5.85 (corr. for 0.56% ash, not alkaline). Re-calcd. for 43.0% C₂H₃O, 7.98% C₅H₆O₄, and 49.02% C₆H₇O₅: C, 49.90; H, 5.57.

The chloroform-insoluble part of the precipitated material was dried at 50° for 18 hr. in a vacuum oven; yield 4.30 g. of tough, somewhat fibrous material. This material was designated Fraction PA-3.

An amount of 9.63 ml. of 0.1004 N sodium hydroxide was required to neutralize the acetic acid produced by distilling 0.1819 g. of Fraction PA-3 with p-toluenesulfonic acid. The material thus contained 22.9% acetyl.

Chloroform was the best solvent for these acetate fractions. Fractions PA-1 and PA-2 were readily dissolved by chloroform and Fraction PA-3 was greatly swollen. Fractions PA-1 and PA-2 were dissolved to a slight extent by acetone. Neither benzene, ethyl ether, nor trichloroethylene showed any tendency to dissolve Fractions PA-1, PA-2, or PA-3.

c. Constituent Sugars of the Polysaccharide Acetate Fractions

A small amount (ca. 20-30 mg.) of each of the polysaccharide acetate fractions was placed in a 25-ml. erlenmeyer flask and warmed with ca. 1 ml. of chloroform. Fractions PA-1 and PA-2
dissolved while PA-3 was greatly swollen. A few drops of methanol followed by a few drops of sodium methylate in methanol (0.5 g. sodium in 100 ml. methanol) were added. The mixtures were warmed and shaken occasionally over a six-hour period.

The insoluble de-acetylated material in each flask was recovered by filtration and hydrolyzed by dissolving in a few ml. of 72% sulfuric acid (ca. 4 hr. required), diluting to ca. 3% sulfuric acid, and heating 4 hr. in a boiling water bath. The almost colorless hydrolyzates were neutralized with barium carbonate, filtered, and electrochromatographed (Figure 22).

Mannose, galactose, arabinose, and a trace of glucose were detected in the hydrolyzates of PA-1 and PA-2. The mannose spot of PA-2 was more intense than that of PA-1. Mannose, glucose, and a trace of galactose were detected in the hydrolyzate of PA-3.
Figure 22. Electrochromatogram of the Hydrolyzates of the Polysaccharide Acetates
IV. DISCUSSION

A. Investigation of an 60/20 : : Ethanol/Water Extract of Green Coffee Bean

1. Inactivation of Enzymes

According to Helferich and Vorsatz (30), the most active carbo-
hydrases in the coffee bean are \( \alpha-D \)-mannopyranosidase, \( \alpha-D \)-
galactopyranosidase, and \( \beta-D \)-galactopyranosidase. A slight amount
of \( \beta-D \)-glucopyranosidase activity is present. The first three are
readily inactivated by heating above 60-70° while the latter is
resistant to heat inactivation and to alcohol inactivation. The
method used in this work for the attempted destruction of enzymes
was boiling the ground green bean with absolute alcohol for 2-3 min.

2. Fractionation of the Extract on Clay (Compare with
Charts I and II and Table 6)

The procedure used in fractionation was similar to that used
by Binkley and Wolf from (61) in the investigation of cane blackstrap
molasses. The method was designed for the separation of sugars and
was based on the work of Lew, Wolf from, and Goepp (39) who investigated
the behavior of sugars on clay.

In this work the main amount of sucrose was found in the 95/5
: : ethanol/water fraction and was removed by crystallization. That
some sucrose was present in the 80/20 fraction was indicated by paper chromatography.

According to the chromatographic scheme of Lew, Wolfrom, and Goepp (41) no simple sugars should appear in the 50/50 ethanol/water fraction or subsequent fractions. Nevertheless, paper chromatograms of the 50/50 and the water fractions both showed sugar spots when sprayed with p-anisidine hydrochloride reagent. The spots were distorted and of low intensity, however.

The presence of glucose was not proven conclusively. A paper chromatogram of the mother liquors from the crystallization of sucrose from the benzene-insoluble substances (Figure 1) indicated the presence of a trace of glucose.

A large amount of chlorogenic acid was present in the 95/5 fraction. The amount of chlorogenic acid was estimated by the ultraviolet adsorption method of Moores, McDermott, and Wood (60). Chlorogenic acid constituted about one-fourth of the 95/5 fraction and about one-half of the 80/20 fraction (Table 6). In the isolation of chlorogenic acid by complexing with caffeine and potassium ions, some difficulty was encountered in removing all of the tartrate ions added; many recrystallizations were required. Although chlorogenic acid was successfully isolated and identified in a small amount of material, it appeared that the isolation could be more easily accomplished with an ion-exchange resin. However, much difficulty, in the form of a black decomposition product, was encountered in the attempt to remove chlorogenic acid from the water-soluble substances.
of the 95/5 fraction by passage through a column of Duolite A-4 (62). This attempt was not further pursued.

Paper chromatograms indicated only a trace of chlorogenic acid in the 50/50 fraction.

Caffeine appeared only in the 95/5 fraction.

Trigonelline was detected in the 80/20 and the 50/50 fractions by electrochromatography. Trigonelline spots quench the fluorescence of the paper when viewed under ultraviolet light and possess a rust-colored fluorescence of their own.

The amino acids were roughly fractionated by the clay column and all the neutral eluent fractions gave positive ninhydrin tests. The acid eluent fractions were not investigated.

3. The Search for Trace Sugars

Because of the large amount of sucrose present in the 95/5 fraction it was not possible to apply enough material to a paper chromatogram to permit detection of trace sugars without overloading with respect to sucrose. One-half of the 95/5 fraction was defatted by extraction of its aqueous solution with benzene in a continuous liquid-liquid extractor. Fats and caffeine were removed by the benzene. Sucrose was removed from the water-soluble material by induced crystallization. The mother liquor from the sucrose crystallization was treated repeatedly with neutral lead acetate to precipitate chlorogenic acid which was removed by filtration. The excess lead ions were removed as lead sulfide and the material remaining was chromatographed on paper (Figure 2). The only definitive
spot found corresponded to sucrose. Trace sugars may have been lost due to adsorption on the precipitate.

4. Chlorogenic and Isochlorogenic Acid

Fischer and Dangshat (82) established that chlorogenic acid

(82) H.O.L. Fischer and Gerda Dangshat, Ber., 65B, 1037 (1932).

is 3-caffeyl quinic acid (I). The structures of quinic acid II and its 3-lactone and 1,4,5-trimethyl quinic acid, and its lactone and amide were known. It was also known that quinic acid formed only one monoacetone derivative (4,5-isoproylidene quinic acid), that chlorogenic acid was a caffeyl quinic acid, and that chlorogenic acid formed a diacetone derivative and did not form a lactone. Therefore, the splitting of methyl penta-O-methylchlorogenate into 3,4-dimethyl caffeic acid and 1,4,5-trimethyl quinic acid lactone (also characterized as the amide) by alkali was a conclusive proof of the structure first proposed by Freudenberg (83).


In 1950 Barnes, Feldman, and White (63) reported the isolation of isochlorogenic acid (III) (5-caffeyl quinic acid) from green coffee beans. Isochlorogenic acid does not form a complex analogous to the crystalline potassium caffeine chlorogenate but is isolated by extraction of acidified green coffee extracts with butyl acetate, a solvent in which chlorogenic acid is nearly insoluble. Neither isochlorogenic acid or any derivative could be obtained crystalline.
The concept that isochlorogenic acid is a position isomer of chlorogenic acid was based on the similarity of the molecular weights, ultimate composition, and the infrared and ultraviolet adsorption spectra.

The variability of the neutralization equivalent and the fact that isochlorogenic yields partially lactonized quinic acid on acid hydrolysis while chlorogenic yields the free acid are indicative of lactonization (free hydroxyl at Position 3). Conductivity increments produced by isochlorogenic and chlorogenic acid in boric acid solution were similar. Since adjacent hydroxyls are necessary for the formation of the boric acid complex it was assumed that Position 4 was free in isochlorogenic as it is in chlorogenic acid.

The choice between Positions 1 and 5 was based on the rates of oxidation of chlorogenic, quinic, and isochlorogenic acids with periodic acid at pH using the method of Pohle, Mehlenbecker, and Cook (84). The oxidation curve and the amount of periodic acid consumed by isochlorogenic acid corresponded to that of chlorogenic acid rather than that of quinic acid. This evidence seemed to indicate Position 5 as the point of attachment of the caffeyl group.

More recently, Descartes de Garcia Paula and Brooks (85)

(84) W. D. Pohle, V. C. Mehlenbecker, and J. H. Cook, Oil and Soap, 22, 115 (1945).

(85) R. Descartes de Garcia Paula and Georges Brooks, Rev. quim. ind., 252, 16 (1953).
examined the tannin substances of mate (Ilex paraguariensis) and of coffee by means of paper chromatography and concluded that they were identical. Two substances were characterized. One was chlorogenic acid ($R_f$ value = 0.7) and the other was thought to be chlorogenic acid which had been oxidized and condensed by the action of polyphenoloxidases ($R_f$ value = 0.9). This latter substance was practically insoluble in cold water, slightly soluble in hot water, and soluble in ethanol. It gave a green color with ferric ions and other reactions of tannins. The substance was referred to as "resinotanol" and was assumed by them to be identical to isochlorogenic acid.

The $R_f$ values of chlorogenic acid (0.39) and isochlorogenic acid (0.54) in 40/19/11 1-butanol/water/ethanol determined in this work are comparable to those of de G. Paula and Brooks. On the basis of the high $R_f$ value, the relatively simple structure for isochlorogenic acid proposed by Barnes, Feldman, and White (63) seems more plausible than an oxidized and condensed chlorogenic acid compound proposed by de G. Paula and Brooks.

B. Investigation of an 80/20 1:1 Ethanol/Water Extract of Roast Coffee Bean

The investigation of the roast coffee bean extract was complicated by the ubiquitous dark brown (yellow fluorescent) decomposition products. There was no evident fractionation of the brown-colored substances by the clay column, and on paper chromatograms yellow-brown streaks were formed rather than discrete spots. A large amount of the brown material was observed not to move at all
on the paper chromatograms (particularly with the 50/50 and water fractions). This material is probably polymeric in nature.

An unidentified substance appears in the paper chromatograms of the 95/5 fraction of roast coffee which is not present in those of the 95/5 fraction from green coffee (see Figure 6 and Figure 8). This spot has a blue-white fluorescence similar to chlorogenic, isochlorogenic, and caffeic acid. The fluorescence is not strong, indicating a moderate to low concentration.

From the intensity of the sugar spots produced by p-anisidine on the paper chromatograms, it is estimated that there is 2-4 times as much sucrose in the roast coffee bean as either glucose or fructose, and that there is probably slightly more fructose than glucose.

The odor characteristics of the fraction solids were distinctive. The water fraction had a pronounced pyridine (and possibly aliphatic amine) odor. The 50/50 fraction had a very mild and highly aromatic coffee aroma. The 80/20 fraction had a more harsh coffee aroma. The odor of the 95/5 fraction was masked by a trace of ethanol, even after lyophilization.

It is significant that no free amino acids were found in the 80/20 : : ethanol/water extract of roast coffee bean. The amount of free amino acids found in the 80/20 : : ethanol/water extract of the green coffee bean was not large, however.
C. Isolation and Separation of the Polysaccharide Fractions

1. Removal of Low Molecular Weight Materials and Proteins

It was desirable, before proceeding with polysaccharide separation, to remove as much as possible of the low molecular weight material (simple sugars, organic acids, lipids, etc.) present in the coffee bean. Some of these materials are readily oxidized and would interfere with the preparation of the holocellulose. In order that the investigation of the polysaccharides might properly tie in with the investigation of the simple sugars, the freshly ground beans were first extracted with 80/20 ethanol/water. None of the extraction procedures accomplishes a clean-cut separation of one type material from another. This was particularly evident with the 80/20 ethanol/water extraction for a not inconsiderable amount of fats dissolved in this medium. The 80/20 ethanol/water extraction served also to reduce the water content of the ground beans and thus facilitate the next step, removal of the lipids.

The efficiency of 2/1 benzene/ethanol in extracting the lipids is worth noting. In about 2½ hr. the fat content of the crushed beans was reduced from ca. 10% to less than 0.5% (actual values, 0.43% by a 72-hr. extraction with ethyl ether and 0.48% by a 72-hr. extraction with petroleum ether). Some non-fat material was also removed, again indicating the overlapping of the extractions.

Having de-fatted the crushed beans, it was possible to carry out a rapid, cold water extraction and remove essentially all of the remaining low molecular weight materials as shown by the amount and
physical appearance of the material removed by two subsequent water
extractions. The first water extract was fractionated by adding
ethanol to produce a 70% ethanol concentration. Slightly more than
one-fourth of the dissolved solids were precipitated by this treatment.
The slimy precipitated material (3.5% of the dry wt. of the original
bean) probably represents most of the protein of the bean.

The removal of proteins was by no means complete for the residue
from the water extraction gave a positive ninhydrin test.

Underwood and Deatherage (86) found that most of the protein

\[ (86) \text{G. E. Underwood and F. E. Deatherage, Food Research,} \]
\[ 17, 419 (1952). \]

which could be extracted from green Santos coffee beans was water-
soluble. They found no prolamines (soluble in 80% ethanol), 3.0%
soluble in water, 3.1% soluble in 10% sodium chloride solution, and
4.9% soluble in 1% sodium hydroxide.

2. Extraction with Dilute Ammonium Oxalate Solution

Not all of the pectic material of plants is extractable with
water. The insoluble part, protopectin (or calcium pectate), was
first removed from plant material by Mangin (87) by extraction with

\[ (87) \text{L. Mangin, Compl. rend. 107, l\textsuperscript{i}4 (1888).} \]
ammonium oxalate solution. Presumably a metathetical reaction takes
place forming soluble ammonium pectate and insoluble calcium oxalate.

An assay of the pectin content of the solids (mostly ammonium
oxalate) from the ammonium oxalate extracts was not made. However,
from the ash content of the residue from the second ammonium oxalate extraction, the maximum amount of ammonium oxalate consumed in the extraction can be calculated and thus also the amount of ammonium oxalate which passed into the extract. The solubility of calcium oxalate is 0.0014 g. per 100 ml. at 95°, but the overwhelming amount of ammonium oxalate present reduces the solubility of the calcium oxalate to a negligible value. Duplicate determinations of the ash content of the residue gave 0.19% and 0.15% ash, as sulfate. Assuming the metal to be calcium, the amount of calcium oxalate in the residue would be about 0.6 g. Thus, all but ca. 0.5 g. of the ammonium oxalate used in the extractions must have gone into the filtrate unchanged. The subtraction of 119.5 g. (wt. of ammonium oxalate which passed into the filtrate) from the weight of solids obtained by lyophilization of the ammonium oxalate extracts leaves 21.4 g. of material (3.0% of the dry wt. of the bean) extracted.

The variety of sugars (galactose, mannose, arabinose, rhamnose or apiose, and probably galacturonic acid) detected in this material by paper chromatography of the hydrolyzate indicates some heterogeneity. Mannose is not ordinarily a constituent of pectins.

3. Holocellulose Preparation

Through all the previous extractions the ground coffee beans retained a characteristic light brown color. The substance responsible for the color is not known. The residue from the final ammonium oxalate extraction still gave a positive ninhydrin test and a lignin value of ca. 2.5%. The validity of the lignin values
for the original bean and the various fractions is uncertain. Analysis of a sample of the "acid lignin" isolated, revealed only 3.07% methoxyl, a low value for lignin. Brauns (88) states that


"lignins isolated from coniferous woods and cereal plants have a methoxyl content of 15-16% and those of deciduous wood have 20.5-21.5%". The carbon and hydrogen values were 59.22% and 7.99%, respectively. Lignins ordinarily have 60-65% carbon and 5.5-6.0% hydrogen.

After the treatment with hot acidic sodium chlorite solution by a modification of the procedure of Whistler and co-workers (52), the residue was almost pure white and gave a negative ferric chloride test, a negative ninhydrin test, and a positive Molisch test. This residue, coffee holocellulose, (ash, 0.91% and 0.94% as sulfate; lignin, 1.77% and 1.82%) represents 40.6% of the dry weight of the original bean.

4. Extraction of the Holocellulose with Alkali

In order to find a suitable potassium hydroxide concentration for the extraction of the hemicelluloses, samples of the holocellulose were extracted with 5%, 10%, and 20% (by weight) aq. potassium hydroxide. The data of Table 1, p. 78, show that the amount of alkali-insoluble material decreases on increasing the alkali concentration from 5% to 10% potassium hydroxide. When the alkali concentration was further increased to 20% the amount of
alkali-insoluble material increased unexpectedly. It is believed that in this concentrated solution there is considerable adsorption of alkali and that due to the nature of the material (coarse, granular) this alkali was not readily removed by the washing procedure used. Another observation supported this conclusion. When samples of the three alkali-insoluble residues (5%, 10%, and 20%) were placed together in a vacuum oven at 70° for 16-18 hr. for moisture determination, the 5%- and 10%-insoluble samples were recovered apparently unchanged (pure white) while the 20%-insoluble sample was brown in color.

In all subsequent extractions 10% aq. potassium hydroxide was used.

The alkali fractions of the holocellulose were hydrolyzed by several methods and the constituent sugars identified by paper chromatography and electrochromatography. The 10% potassium-insoluble residue was found to contain galactose, mannose, arabinose, glucose, and an inconclusively identified, slow-moving spot (probably a uronic acid).

5. Characterization of the Alkali-Insoluble Holocellulose

a. Solubility in Cuprammonium Solution

Schulze (3) and Gilson (23) both found the insoluble polysaccharides of coffee could be dissolved in cuprammonium solution (after a preliminary mild hydrolysis). Furthermore, Gilson claimed to have precipitated cellulose by the slow introduction of carbon dioxide leaving a mannose-yielding polysaccharide in solution.
When the coffee holocellulose was let stand in cuprammonium solution for ca. 26 hr., only a small amount of material was dissolved (1.1 g. by isolation). No particular precautions were taken to exclude oxygen in this experiment. Thus, it is possible that some degradation may have occurred.

Hydrolysis of the cuprammonium-soluble material yielded a hydrolyzate which contained mainly mannose plus some glucose and a trace of galactose (electrochromatography). This indicates that some separation of the polysaccharides might be effected by this method without the undesirable (from a structural study standpoint) pre-hydrolysis.

b. Partial Hydrolysis of the Holocellulose

It was found that arabinose is the main pentose present in the coffee holocellulose. (A trace of xylose was present.) Moreover, it was found that, within the limits of detection by paper chromatography, all of the arabinose units could be split out of the alkali-insoluble holocellulose by refluxing 15 min. with 1.5% sulfuric acid. This result was not unexpected since arabinose commonly occurs in nature in the labile furanose ring form.

The galactose residues in the holocellulose are also rather readily removed by boiling with 1.5% sulfuric acid for 15 min. This is indicated by presence of a prominent galactose spot in the chromatogram (Figure 18) of the soluble products and also by the failure to isolate galactose 1-methyl-1-phenylhydrazone from the insoluble products by the method of Hirst, Jones, and Wood (74) for
the estimation of galactose.

c. Estimation of the Sugar Ratio in Alkali-
Insoluble Coffee Holocellulose

Although it was recognized that the alkali-insoluble holocellulose was a mixture of polysaccharides, it was considered desirable to know the amounts of the constituent sugars in this important fraction. The attempts to estimate the constituent sugars met with varying success.

The estimation of anhydroarabinose was carried out with the least amount of manipulation and followed a well-standardized procedure for converting the pentosan to furfural phloroglucide which was isolated and weighed. The weight of anhydroarabinose present was found from Kröber's tables (73). The average of three estimations was 6.7% anhydroarabinose. This compares favorably with the value of 6.72% pentosan found by Schulze (3) in a water-, alcohol-, and dilute ammonium hydroxide-insoluble residue which he isolated from coffee beans.

The failure to obtain galactose 1-methyl-1-phenylhydrazone from either the 1.5% sulfuric acid or the 72% sulfuric acid hydrolyzed residue from alkali-insoluble holocellulose is not readily explained. The chromatographic evidence for galactose seems overwhelming, but this point needs further confirmation. To be conclusive the identification should be put on a crystalline basis.

The values (45.2% and 46.3%) for the amount of mannose (isolated as the phenylhydrazone) in the alkali-insoluble holocellulose represent minimum figures for a trace of mannose is removed by the 1.5%
sulfuric acid hydrolysis.

No attempt was made to estimate the amount of glucose or the small amount of uronic acid present. Glucose could be estimated by difference if the amounts of galactose and uronic acids were determined.

d. Acetylation of Alkali-Insoluble Coffee Holocellulose

The pyridine-acetic anhydride procedure was unsuitable for the acetylation of the holocellulose even though the material could be swollen somewhat by stirring in hot formamide according to the method of Carson and Maclay (78).

In view of the difficulty encountered in trying to acetylate the holocellulose with pyridine-acetic anhydride, the ease with which acetylation was accomplished by the acetic acid-trifluoro-acetic anhydride method was remarkable. Even at ca. 0° most of the holocellulose dissolved in the reaction mixture within an hour. Although the reaction mixture was homogeneous at the conclusion of the reaction (5 hr.), about half of the isolated acetylated material (Fraction PA-3) was insoluble in common solvents (chloroform, benzene, acetone, trichloroethylene, and ethyl ether). This fraction was greatly swollen by chloroform. The acetyl value (22.9%) of this material was low.

The other half of the acetylated material was highly acetylated (Fractions PA-1 and PA-2, 42.0% and 39.2% acetyl, respectively). It was readily dissolved by chloroform and partly dissolved by acetone. Benzene and ethyl ether had no visible solvent effect.
upon this material.

At least a partial fractionation of polysaccharides was achieved by acetylation of the holocellulose. Electrochromatography (Figure 22) of the de-acetylated and hydrolyzed polysaccharide acetate fractions revealed the presence of arabinose, galactose, mannose, and a trace of glucose in Fractions PA-1 and PA-2. On the other hand mannose, glucose, and a trace of galactose were detected in the hydrolyzate of PA-3.
V. SUMMARY

A. Green Coffee Extract

1. An 80/20 : : ethanol/water extract of green coffee beans was fractionated on clay using graded aqueous ethanol developers.

2. The 95/5 : : ethanol/water fraction contained sucrose, chlorogenic acid, isochlorogenic acid, caffeic acid, caffeine, and some fats. Paper chromatograms indicated the presence of glucose.

3. The 80/20 : : ethanol/water fraction contained chlorogenic acid, isochlorogenic acid or caffeic acid or both, trigonelline, a small amount of sucrose and seven amino acids: glycine, α-alanine, γ-aminobutyric acid, proline, valine, leucine (or isoleucine), and tyrosine.

4. The 50/50 : : ethanol/water fraction contained trigonelline and six amino acids: aspartic acid, glutamic acid, serine, asparagine, α-alanine, and γ-aminobutyric acid.

5. Techniques were developed for the detection of caffeine on paper chromatograms and clay columns.

6. Isochlorogenic acid was isolated from the green coffee bean and characterized chromatographically.

B. Roast Coffee Extract

1. An 80/20 : : ethanol/water extract of roast coffee bean was
fractionated on clay, using graded ethanol/water developers.

2. The 95/5 % ethanol/water fraction contained sucrose, glucose, fructose, chlorogenic acid, isochlorogenic acid or caffeic acid or both, and caffeine.

3. The 80/20 % ethanol/water fraction contained chlorogenic acid, isochlorogenic acid or caffeic acid or both, and trigonelline.

4. The 50/50 % ethanol/water fraction and the water fraction probably contained polymeric materials.

5. Free amino acids were absent in the roast coffee.

C. Polysaccharide Fractions of Green Coffee

1. Ground green coffee bean has been fractionated by successive extraction with 80/20 % ethanol/water, 2/1 % benzene/ethanol, water, 0.5% aqueous ammonium oxalate, acidified aqueous sodium hypochlorite, and 10% potassium hydroxide.

2. The water-soluble polysaccharide material gave rhamnose (or apiose), galactose, and arabinose on hydrolysis.

3. The ammonium oxalate extracts gave galactose, arabinose, mannose, rhamnose (or apiose), and two unidentified carbohydrates on hydrolysis.

4. The 10% potassium hydroxide-soluble fraction gave rhamnose (or apiose), xylose, arabinose, galactose, a trace of mannose, and several unidentified carbohydrates (probably oligosaccharides) on hydrolysis. The 10% potassium hydroxide-soluble fraction was mainly of the hemicellulose-B type, not precipitated by acidification but completely precipitated by addition of ethanol.
5. The 10% potassium hydroxide-insoluble residue gave glucose, arabinose, galactose, mannose, and one inconclusively identified carbohydrate, probably a uronic acid, on hydrolysis.

6. The amount of arabinose in the 10% potassium hydroxide-insoluble holocellulose was estimated by the phloroglucinol method. The average of three determinations was 6.7%.

7. The amount of mannose in the 10% potassium hydroxide-insoluble holocellulose was estimated by preparation of the phenylhydrazone. The average of two determinations was 45.8%.

8. D-mannose was identified in the 10% potassium hydroxide-insoluble residue, not hydrolyzed by 1.5% sulfuric acid, as the β-pentaacetate and as the phenylhydrazone.

9. D-glucose was identified in the 10% potassium hydroxide-insoluble residue, not hydrolyzed by 1.5% sulfuric acid, as the β-pentaacetate.

10. The 10% potassium hydroxide-insoluble holocellulose was acetylated with acetic acid-trifluoroacetic anhydride. Fractionation of the acetylated holocellulose afforded a chloroform-soluble part containing mainly mannose, galactose, and arabinose plus a trace of glucose and a chloroform-insoluble part containing mannose and glucose plus a trace of galactose.
Ground Green Coffee Bean (500 g.)

Extraction with
80/20 \( \text{C}_2\text{H}_5\text{OH}/\text{H}_2\text{O} \)

Aqueous Ethanol Extract of Green Coffee Bean (Yield, 90.0 g.)

Fractionation on Fuller's Earth Clay

<table>
<thead>
<tr>
<th>Percolation</th>
<th>Percolation</th>
<th>Percolation</th>
<th>Percolation</th>
<th>Percolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>of 5 liters of 95/5 ( \text{C}_2\text{H}_5\text{OH}/\text{H}_2\text{O} ) (yield, 75.1 g.)</td>
<td>of 6 liters of 80/20 ( \text{C}_2\text{H}_5\text{OH}/\text{H}_2\text{O} ) (yield, 10.5 g.)</td>
<td>of 5.5 liters of 50/50 ( \text{C}_2\text{H}_5\text{OH}/\text{H}_2\text{O} ) (yield, 2.6 g.)</td>
<td>of 4 liters of water (yield, 1.3 g.)</td>
<td>of 3 liters of 0.1 N HCl (Estimated yield, 0.7 g.)</td>
</tr>
</tbody>
</table>

Chart I. Flow Sheet for the Fractionation of an 80/20 Ethanol/Water Extract of Green Coffee Bean
Chart II. Flow Sheet of the Investigation of the 95/5 :: Ethanol/Water Fraction

95/5 :: Ethanol/Water Fraction from the Fractionation of an 80/20 :: Ethanol/Water Extract of Green Coffee Bean

(800 ml.) Induced Crystallization

- Mother Liquor
- Sucrose

Concentration and Ether Extraction

- Ether-Insoluble Substances
- Ether-Soluble Substances

- Lyophilize
- Ether-Insoluble Solids
- Dissolve in water and extract with benzene
- Ether-and Benzene-Insoluble Substances
- Crude Caffeine
- Recrystallize
- Caffeine

Benzene-Insoluble Substances

Benzene-Soluble Substances

- Lyophilize
- Benzene-Insoluble Solids
- Induced Crystallization
- Mother Liquor
- Sucrose and Chlorogenic acid
Table 6. Fractionation of 90 Grams of Material from an 80/20 ✦
Ethanol/Water Extract of 500 Grams of Green Coffee
Beans on Florex XXX® with Graded Ethanol/Water Developers

<table>
<thead>
<tr>
<th>Developer</th>
<th>Wt. of material eluted from column, g.</th>
<th>Sub-fractions of the eluted material</th>
<th>Substances identified</th>
<th>Wt. of substances identified, g.</th>
<th>% of dry bean of substances identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 liters of 95/5 ✦</td>
<td>75.1 (= 17.8% of the dry bean)</td>
<td>Ether-soluble substances (12.8 g.)</td>
<td>Sucrose</td>
<td>24.9</td>
<td>5.53</td>
</tr>
<tr>
<td>5 liters of ethanol/water</td>
<td>75.1 (= 17.8% of the dry bean)</td>
<td>Chlorogenic acid</td>
<td>22.9</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Caffeine</td>
<td>3.1</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fats</td>
<td>12.9</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amino acids</td>
<td>trace</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>6 liters of 80/20 ✦</td>
<td>10.5 (=2.3% of the dry bean)</td>
<td>Benzene-soluble substances (17.8 g.)</td>
<td>Sucrose</td>
<td>present</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chlorogenic acid</td>
<td>4.6</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trigonelline</td>
<td>present</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glycine</td>
<td>present</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Alanine</td>
<td>present</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Aminobutyric acid</td>
<td>present</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proline</td>
<td>present</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Valine</td>
<td>present</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leucine</td>
<td>present</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tyrosine</td>
<td>present</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>5.5 liters of 50/50 ✦</td>
<td>2.6 (= 0.6% of the dry bean)</td>
<td>Trigonelline</td>
<td>present</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aspartic acid</td>
<td>present</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glutamic acid</td>
<td>present</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Serine</td>
<td>present</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Asparagine</td>
<td>present</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Alanine</td>
<td>trace</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Aminobutyric acid</td>
<td>trace</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>4 liters of water</td>
<td>1.3 (= 0.3% of the dry bean)</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>7 liters of dilute aq. HCl</td>
<td>0.6 (= 0.1% of the dry bean)</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
</tbody>
</table>

*A Fuller's earth clay, 5 parts Florex XXX to 1 part Celite No. 535 (wt. ratio).

b -Alanine and -aminobutyric acid were estimated to be present in about equal amounts and to constitute together about one-half of the total amount of amino acids in the 80/20 ✦ ethanol/water fraction.
Ground Roast Coffee Bean (500 g.)

Extraction with
80/20 :: C₂H₅OH/H₂O

Aquous Ethanol Extract of Roast Coffee Bean (Yield, 80.0 g.)

Fractionation on Fuller's Earth Clay

Percolation of
7 liters of
95/5 :: C₂H₅OH/H₂O
(yield, 59.64 g.)

Percolation of
6 liters of
80/20 :: C₂H₅OH/H₂O
(yield, 11.40 g.)

Percolation of
5 liters of
50/50 :: C₂H₅OH/H₂O
(yield, 5.94 g.)

Percolation of
4 liters of
water
(yield, 1.94 g.)

Percolation of
6 liters of
0.1 N HCl
(yield, est., 1.1 g.)

Chart III. Flow Sheet for the Fractionation of an
80/20 :: Ethanol/Water Extract of
Roast Coffee Bean
Table 7. Fractionation of 80 Grams of Material from an 80/20 \( \times \)  
Ethanol/Water Extract of 500 Grams of Roast Coffee 
Beans on Florez XXX with Graded Ethanol/Water Developers

<table>
<thead>
<tr>
<th>Developer</th>
<th>Wt. of material eluted from column, g.</th>
<th>Substances identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 liters of 95/5 ( \times ) ethanol/water</td>
<td>59.64 (= 12.61% of the dry bean)</td>
<td>Sucrose, Glucose, Fructose, Chlorogenic acid, Caffeine, Isochlorogenic acid or both, Caffeic acid</td>
</tr>
<tr>
<td>6 liters of 80/20 ( \times ) ethanol/water</td>
<td>11.34 (= 2.4% of the dry bean)</td>
<td>Trigonelline, Chlorogenic acid, Isochlorogenic acid or both, Caffeic acid</td>
</tr>
<tr>
<td>5 liters of 50/50 ( \times ) ethanol/water</td>
<td>5.94 (= 1.26% of the dry bean)</td>
<td>Polymeric material</td>
</tr>
<tr>
<td>4 liters of water</td>
<td>1.94 (= 0.41% of the dry bean)</td>
<td>Polymeric material</td>
</tr>
<tr>
<td>6 liters of 0.1 N HCl</td>
<td>ca. 1.1 (= 0.22% of the dry bean)</td>
<td></td>
</tr>
</tbody>
</table>

\*A Fuller's earth clay, 5 parts Florez XXX to 1 part Celite No. 535 (wt. ratio).\*
Chart IV  Flow Sheet for the Fractionation of the Polysaccharides of the Green Coffee Bean (g./100 g., dry weight basis)

100.0 g. Ground Green Coffee

\[\text{80/20} \text{ : : Ethanol/Water}\]

- Filtrate: 15.5 g.\(^a\)
- Residue (calcd. 84.5 g.)

\[\text{2/1} \text{ : : Benzene/Ethanol}\]

- Extract: 17.1 g.\(^b\)
- Residue (66.8 g.)\(^b\)
  (Calcd. 67.4 g.; recovery, 99%)

- Water Extraction (3 times)

- Filtrate: 15.2 g.\(^a\)
- Residue (51.3 g.)\(^b\)
  (Calcd. 51.6 g.; recovery, 99%)

- 0.5% Ammonium Oxalate Extraction (2 times)

- Filtrate: 3.0 g.\(^{ab}\)
  (pectin)
- Residue (46.7 g.)\(^{ab}\)
  (Calcd. 48.4 g.; recovery 97%)

- Acidified Sodium Chlorite
- Filtrate: 6.2 g.\(^a\)
  (calcd. 6.2 g.)
- Residue (40.6 g.)\(^a\)
  (holocellulose)

- 10% KOH Extraction
- Filtrate: 36.0 g.\(^a\)
  (recovery, 90%)

- Neutralize

- Centrifugate

- Ethanol (3 vol.)
- Hemicellulose-B (2.9 g.)
- Hemicellulose-A (0.2 g.)\(^a\)

Total Analysis, 96.1 g.

\(^a\) By isolation.
\(^b\) By analysis.
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AUTOBIOGRAPHY

I, Richard Allan Plunkett, was born in Miami County, Ohio, August 12, 1929. I attended the Newton Township Rural Schools and was graduated in May, 1947. In September, 1947, I enrolled in Manchester College at North Manchester, Indiana, from which I received the degree Bachelor of Arts in May, 1951.

In September, 1951, I enrolled in the Graduate School of The Ohio State University. I was appointed research assistant on a Nestlé Company project for the study of coffee oil under the Ohio State University Development Fund. In September, 1952, the Nestlé Company project was placed with the Ohio State Research Foundation and I was appointed Research Fellow. In December, 1952, I received the degree Master of Science from The Ohio State University.

The subject of the Nestlé Company project was changed to the investigation of the carbohydrates of the coffee bean in October, 1953. I held the position of Research Fellow on this project from October, 1953, to September, 1955, while completing the requirements for the degree of Doctor of Philosophy.