Identification and Quantification of the Free Sugars in Cocoa Beans

Gary A. Reineccius,[1] David A. Andersen,[2] Terrance E. Kavanagh,[3] and Philip G. Keeney*

Glc of the silylated sugars fraction of cocoa beans revealed fructose, sorbose, glucose, sucrose, inositol, mannitol, a pentitol, and traces of two unidentified sugars. The same sugars were present in all samples, irrespective of geographic origin. Relative concentrations were, however, quite different, even among lots of the same type of bean. Most of these differences were attributed to harvesting, fermenting, and drying variables. Unfermented Sanchez beans were 1% by wt sucrose, but fermented Bahia and Ghana beans averaged only 0.05 and 0.12% sucrose, respectively. Fermented varieties contained 2 to 16 times more fructose than glucose. The preferential consumption of the glucose moiety of sucrose during fermentation has implications important to the development of chocolate flavor during roasting. Results suggest that absorbed and occluded water-soluble constituents from the pulp contribute to the reducing sugars content of cocoa beans.

Although cocoa beans contain only small amounts of sugars, nonenzymatic browning reactions are, nevertheless, essential to the development of the typical aroma of chocolate. This is evidenced by the 30 pyrazines, 10 pyrroles, and 15 furans identified in the aroma fraction (van Elzakker and van Zutphen, 1961; Bailey *et al.*, 1962; Dietrich *et al.*, 1964; Marion *et al.*, 1967; Flament *et al.*, 1967; Rizzi, 1967; van der Wal *et al.*, 1968, 1971; van Praag *et al.*, 1968).

Cerbulis (1954), using paper chromatography, confirmed the work of others and identified D-fructose, D-glucose, sucrose, raffinose, stachyose, and D-galactose in Caracas cocoa beans. In a later publication, Cerbulis (1956) added melibiose, manninotriose, mesoinositol, and an unidentified sugar alcohol to the list, and he speculated on the presence of several others.

Quantitative data for individual sugars have not been reported, but measurements of reducing sugars and total sugars have been made, most recently by Rohan and Stewart (1966b, 1967). Values for reducing sugars ranged from 0.34 to 1.38% by weight of cotyledon in commercial cocoa beans obtained from the major producing countries. Total sugars ranged from 0.39 to 3.48%. The postharvest fermentation process is a major factor affecting the sugars; sucrose disappears almost completely during fermentation (Knapp, 1937), but the fate of the reducing sugars is less clear. Rohan and Stewart (1967) revealed a rapid formation of reducing sugars in African beans, which reached a maximum concentration after only 2 or 3 days of fermentation in heaps. Thereafter, the concentration of reducing sugars remained fairly constant and then decreased slightly near the end of the fermentation process.

While fermentation is essential to the formation of flavor precursors, the typical flavor of chocolate does not develop until the cocoa beans have been roasted. Rohan and Stewart (1966b) reported a nearly complete destruction of reducing sugars and a significant reduction in total sugars during the roasting process. These changes in the sugars of cocoa beans are, undoubtedly, important variables affecting the formation of volatile compounds from nonenzymatic browning reactions.

Methods used for the qualitative and quantitative determination of sugars have not adapted well to the analysis of chocolate products because of the small quantities present, as well as the difficulties involved in obtaining samples sufficiently free from interfering substances. Sugars analysis by gas chromatography of trimethylsilyl (TMS) ether derivatives (Sweeley *et al.*, 1963; Richey *et al.*, 1964) offered possibilities for overcoming some of these difficulties. Using these procedures, an investigation was undertaken to collect additional information about the sugars in cocoa beans and how they are affected by fermenting and processing variables.

EXPERIMENTAL

Extraction of Sugars. The procedure used to isolate carbohydrates from cocoa beans was basically that of Andersen (1968). Ten grams of beans and 40 mg of rhamnose (internal standard) were pulverized for 5 min in a Waring Blendor with 150 ml of water:methanol (80:20). The sides of the Blendor were rinsed several times with distilled water during grinding. The suspension was then transferred to a 250-ml bottle and centrifuged for 15 min at 2000 rpm. The supernatant was decanted, adjusted to pH 10 with 1 *N* KOH, and 20 ml of saturated lead acetate solution was added to precipitate the polyphenols. The polyphenol–lead complex was removed from solution by centrifugation for 10 min at 2000 rpm. The clear supernatant was then passed through a 10 × 1.5 cm bed of Dowex 50-WX8 (Na^+ form) cation and a 10 × 1.5 cm bed of Dowex 2-X8 anion ion exchange resins. The Na^+ form of the cation resin was necessary to prevent sucrose hydrolysis on the column.

The eluant from the ion exchange column was freeze dehydrated in a Stokes Freeze Drier without application of external heat in order to prevent browning or caramelization reactions in nearly dry samples. The residue was transferred with distilled water to a 20-ml vial and was again freeze dried. Five milliliters of anhydrous pyridine (stored over KOH pellets) was added to the vial, and after shaking well, a 1-ml aliquot was transferred to another vial for analysis. This extraction separated the sugars from the pyridine-insoluble proteins and NaCl from the ion exchange columns. The sugars actually comprised only a small portion of the freeze-dried residue.

Sugars in the 1-ml sample were converted to trimethylsilyl ethers (TMS) by adding 0.5 ml of hexamethyldisilazane and

Division of Food Science and Industry, Borland Laboratory, The Pennsylvania State University, University Park, Pa. 16802.

[1] Present address: Department of Food Science and Industries, University of Minnesota, St. Paul, Minnesota.

[2] Present address: Carnation Research Laboratories, Van Nuys, California.

[3] Present address: Carlton & United Breweries Ltd., Melbourne, Australia.

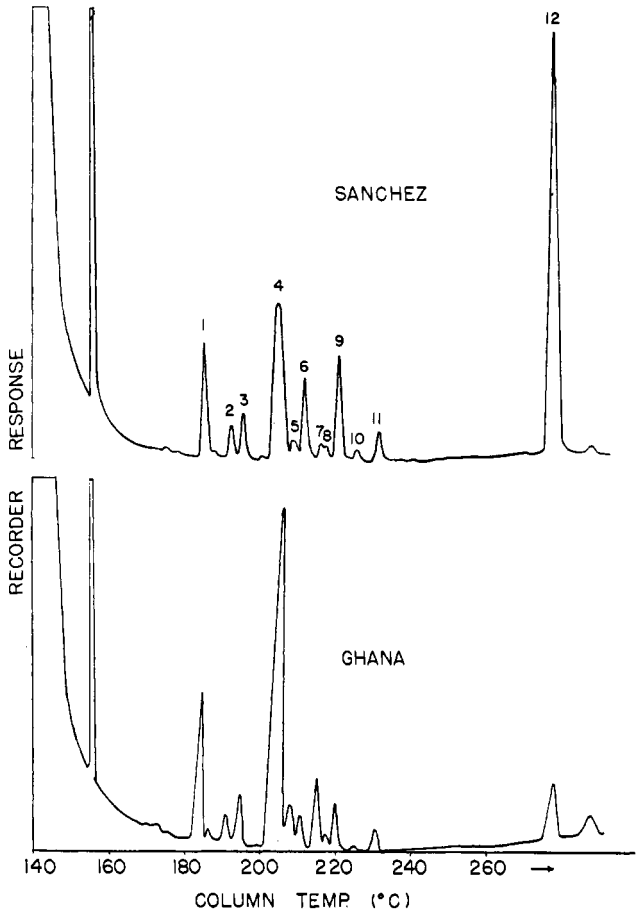

Figure 1. Glc of free carbohydrates (TMS ethers) from Sanchez (unfermented) and Ghana (fermented) cocoa beans. Peaks 1 and 2, rhamnose internal standard; 3, pentitol; 4, α- and β-fructose; 5, sorbose; 6, α-glucose; 7, unknown; 8, mannitol; 9, β-glucose; 10, unknown; 11, inositol; 12, sucrose

0.3 ml of trimethylchlorosilane. The TMS sugar derivatives were chromatographed within 4 hr after silylation. While the hexose derivatives were stable for longer periods, sucrose content decreased after about 4 hr and extraneous peaks appeared on the chromatograms.

Identification of Sugars. With mass spectrometry it is possible to differentiate between classes of carbohydrates (*e.g.*, keto sugars, amino sugars, deoxy sugars) but not between members of a class. Fortunately, anomers and epimers of a class are easily resolved by glc (Sweeley *et al.*, 1963). Thus, mass spectra were used to assign an unknown to a class and glc retention times established which member the unknown peak represented (Reineccius *et al.*, 1970).

An LKB 9000 combined glc–ms was used for the collection of mass spectra. Separation of TMS derivatives was obtained on a 1.83-m × 0.64-cm glass column packed with the 5% SE-30 on 80/100 mesh Hi-Performance AWDMCS treated Chromosorb G. Program conditions were 120 to 260°C at 6°C/min using helium at 15 ml/min. The LKB 9000 has as a detection system, a total ion current recording of the gas chromatographic eluants. Flash heater and molecular separator temperatures were 280°C. Mass spectra were obtained using a constant accelerating voltage of 3500 Volts with 70 eV energy and a scanning time of 7 sec over an *m/e* range of 4 to 600.

After the sugars had been assigned to classes by mass spectrometry, retention times of members of a class were compared to reference compounds using a Hewlett-Packard Model 5750B gas chromatograph equipped with a flame ionization detector. The column, 6-ft × 1/8-in. stainless steel containing the same packing material employed for mass spectrometry, was programmed from 140 to 260°C at 2°C/min with a carrier gas (N_2) flow of 30 ml/min and an injection port temperature of 280°C.

Quantitative Measurement of Sugars. The Hewlett-Packard machine was used for the collection of quantitative data, with rhamnose serving as the internal standard. Peak area was calculated by the triangulation method (height × width at half height). Since the various sugars responded differently in the same ionization detector (Richey *et al.*, 1964), it was necessary to establish a standard curve (peak area *vs.* concentration) for each cocoa sugar relative to the internal standard. For each sugar, detector response was linear over the concentration range examined.

Table I. Composition of the Sugars Fraction of Unroasted Cocoa Beans

Sugar	Cocoa bean variety: Sanchez	Arriba	Bahia	Ghana
	% in Total free sugars			
Pentitol	2.4	4.5	2.7	3.0
Fructose	19.4	21.8	52.3	57.0
Sorbose	2.4	3.3	9.8	6.1
α and β-Glucose	18.5	14.0	3.3	8.2
Mannitol	2.2	2.3	21.4	8.9
Unknown	...	...	...	...
Unknown	0.9	1.2	2.1	0.5
Inositol	1.6	1.5	4.2	2.3
Sucrose	53.6	51.4	4.2	14.0
Total reducing	40.3	38.2	65.4	71.3
	mg/100 g of beans			
Sucrose	1000	683	55	120
Reducing sugars	747	510	855	612
Total sugars	1856	1332	1308	858

RESULTS AND DISCUSSION

The chromatograms presented in Figure 1 show both the differences and similarities found between cocoa beans from a producing country where fermentation is traditionally practiced (Ghana) and a country where beans generally are not fermented (Sanchez beans, Dominican Republic). The same peaks are present but relative proportions are strikingly different. This general statement applies to all of the comparisons made among beans of diverse geographic origin. In addition, to the Ghanian and Dominican samples, beans from Samoa, Nigeria, Ivory Coast, Trinidad, Mexico (Tabasco), Ecuador (Arriba), and Brazil (Bahia) were included in the study.

Using the previously described techniques, the following compounds were identified (Figure 1): pentitol (peak 3); fructose (peak 4); sorbose (peak 5); α-glucose (peak 6); mannitol (peak 8); β-glucose (peak 9); inositol (peak 11); and sucrose (peak 12), leaving two unknowns (peak 7 and 10). The mass spectrum of peak 7 closely resembled that of a 2-deoxy hexose, but this class of compounds had considerably shorter glc retention times than our unknown. While the mass spectra of peak 10 indicated it was carbohydrate, the spectra could not be associated with any class we have examined.

Quantitative data covering four varieties of cocoa beans are recorded in Table I. Differences shown reflect the in-

fluence of variables associated with harvesting, fermenting, and drying practices. Bahia and Ghana beans, which are usually fermented for several days, contained very little sucrose. This is in marked contrast to the lightly-fermented Arriba and unfermented Sanchez beans, in which sucrose concentration was 10 to 20 times higher. While sucrose has not heretofore been determined directly, evidence is ample (Rohan and Stewart, 1967) that sucrose concentration diminishes during fermentation.

Somewhat surprising were the large differences found between fructose and glucose, especially in the well-fermented samples. Fructose–glucose ratios were 16:1 for Bahia and 7:1 for Ghana beans. For lightly-fermented Arriba the ratio was less than 2:1, and fructose and glucose concentrations were essentially the same in unfermented Sanchez beans. The glucose moiety was either preferentially metabolized or polymerized as sucrose was hydrolyzed during fermentation. The apparent correlation between fermentation and the degree to which the ketoses dominate the reducing sugar fraction has special significance to Maillard browning during the roasting of cocoa beans to develop chocolate flavor. This aspect is discussed in greater detail in an accompanying paper dealing with pyrazine formation in cocoa beans (Reineccius *et al.*, 1972).

Beans examined in this study were from commercial shipments of unknown history and it had to be assumed that they were reasonably representative of beans exported from Ghana, Brazil, Ecuador, and the Dominican Republic. The variableness to be expected for a particular type of bean cannot be established until a large number of samples covering several production years have been analyzed.

From the foregoing it was obvious that conclusions concerning changes in the sugars during fermentation needed support from data collected on beans which had gone through a verified fermentation process. Through the cooperation of V. C. Quesnel, University of West Indies, Trinidad, beans were provided which had been fermented (sweat box) 0, 3, and 7 days under carefully controlled conditions.

Analysis of the Trinidad samples (Table II) revealed that the sucrose was almost totally consumed during the first 3 days of fermentation. This confirms Rohan and Stewart's (1967) conclusion, based on their indirect evidence, that most of the sucrose will have been hydrolyzed midway through the fermentation period.

The sugars profile of unfermented beans and the fructose–glucose ratio for fermented samples in the Trinidad study deserve special mention. Only traces of sugars other than sucrose were detected in beans which had not been fermented. This is in contrast to the supposedly unfermented Sanchez beans which contained a significant quantity of reducing sugars (Table I). The unfermented Trinidad sample had been washed with water shortly after opening of the pods to remove mucilagenous pulp. The sugar data, therefore, can be considered representative of the sugar profile as it exists in beans when the pods are opened. Sanchez beans are not washed prior to sun drying. Their relatively high reducing sugar content suggests that some fermentative reactions take place in "unfermented" Sanchez beans between opening of the pods and final drying.

The fructose–glucose ratio for the well-fermented Trinidad beans was approximately the same, 2:1, recorded for lightly-fermented Arriba beans (Table I). One might have expected fructose's dominance to be of the order found for well-fermented Bahia and Ghana beans. This suggests that other variables, in addition to fermentation time, will have a profound influence on the sugar profile in cocoa beans. A sweat box was used as a fermenter in the Trinidad trial. Most likely, the Ghana beans were fermented in heaps. Other differences in handling practices among the beans included in this investigation cannot be listed with any degree of certainty.

Table II. Changes in the Sugars of Trinidad Beans during Fermentation

Sugar	Unfermented, mg/100 g	Fermented 3 day mg/100 g	Fermented 3 day wt %	Fermented 7 day mg/100 g	Fermented 7 day wt %
Pentitol	Trace	Trace	...	Trace	...
Fructose	Trace	454	53.0	260	46.6
Sorbose	Trace	40	4.7	44	7.9
α and β-Glucose	Trace	224	26.1	214	25.0
Unknown	Trace	Trace	...	Trace	...
Mannitol	Trace	80	9.3	40	7.2
Unknown	Trace	Trace	...	Trace	...
Inositol	Trace	60	7.0	Trace	...
Sucrose	1580	Trace	...	Trace	...
Total reducing	Trace	858	...	558	...

Fermentation is accepted as a critical step in the development of the precursors which are converted to chocolate flavor compounds when cocoa beans are roasted. The sugars, especially the reducing sugars, are recognized as an important precursor class. The finding of only minute traces of sugars other than sucrose in washed, unfermented Trinidad beans raised questions concerning their origin. Specifically, could the mucilagenous pulp from the pod, which contains 15% solids, contribute any of the sugars measured as being indigenous to the cotyledon itself? As reported by Chatt (1953), beans become swollen by absorption of moisture during fermentation and shell tissues become saturated with mucilage. Furthermore, residues remaining from the pulp after completion of fermentation would be retained at the surface of the bean mass and could, therefore, affect chemical and physical properties, both desirable and undesirable.

To determine if sugars from the pulp might possibly contribute to the analytical values obtained for commercial samples of cocoa beans, 50 g of Ghana beans were scrubbed with water in a sonic cleaning apparatus to dissolve sugars adsorbed near the surface of the beans. The water was changed several times. The beans were then dried overnight in a stream of air at room temperature. Inspection of the beans before and after drying did not give any indication that moisture might have penetrated the interior of the bean to leech out sugars. Analysis for sugars revealed that approximately 50% of the sugars were removed by the washing action. This suggests that, depending on the treatments the beans receive, pulp sugars can be retained and contribute to the sugars fraction of cocoa beans. This finding requires further study in respect to fermentation variables which influence the retention of sugars from the pulp. Rohan and Stewart (1966b) concluded that a deficiency of reducing sugars is an especially important factor limiting the development of optimum chocolate flavor during roasting.

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Factors Affecting the Concentration of Pyrazines in Cocoa Beans

Gary A. Reineccius,[1] Philip G. Keeney,* and Wendy Weissberger

Glc of the pyrazine fraction from roasted cocoa beans yielded nine well resolved peaks which could be quantitated. When beans from several producing countries were roasted under identical conditions, pyrazines generated varied between 142 μg/100 g of beans and 698 μg/100 g. The potential for generating pyrazines was greatest in samples from countries where beans are traditionally fermented. Tetramethylpyrazine, trimethylpyrazine, and pyrazines under a peak representing a mixture of 2-ethylpyrazine, 2,5-dimethylpyrazine, and 2,6-dimethylpyrazine were present in the highest concentrations. In fermented cocoa beans, pyrazine concentration increased rapidly during roasting to a near maximum value which did not change during extended roasting. Results indicate that fermentation influences the rate of formation and final concentration of pyrazines in roasted beans primarily through its effect on the free sugars. Ketoses dominated the sugars fraction (62% of total) in well fermented beans compared to 21% in nonfermented varieties. Tetramethylpyrazine was the only pyrazine detected in unroasted beans and then only in fermented samples.

Following the early patent reference (Reichstein and Staudinger, 1928) to pyrazines in coffee, Davison and Wiggens (1956) discovered pyrazine and pyridine bases in an abnormally treated batch of ammoniated molasses. Eight years later Dietrich *et al.* (1964) found 2,6-dimethylpyrazine and tetramethylpyrazine in chocolate liquor. Shortly thereafter pyrazines were identified in coffee (Reymond *et al.*, 1966; Viani *et al.*, 1965), potato chips (Deck and Chang, 1965), and peanuts (Mason *et al.*, 1966). This class of compounds is now recognized as an important contributor to flavor in certain highly heated food systems, especially when processing involves roasting (chocolate, coffee, and nut products). Since the original discovery, 30 pyrazines have been identified in chocolate. These include: Dietrich *et al.* (1964): 2,6-dimethyl; tetramethyl. Rizzi (1967): methyl; 2,3-dimethyl; 2,5-dimethyl; 2-methyl-5-ethyl; trimethyl; 2,5-dimethyl-3-ethyl; 2,6-dimethyl-3-ethyl. Marion *et al.* (1967): 2-methyl-6-ethyl. Flament *et al.* (1967): 2,5-dimethyl-3-propyl; 2,5-dimethyl-3-isoamyl; 2,3-dimethyl-5-isoamyl; 2,3-dimethyl-5-(2-methylbutyl); 2-ethyl-3,5,6-trimethyl; 2-isoamyl-3,5,6-trimethyl; 2-(2-methylbutyl)-3,5,6-trimethyl. van Praag *et al.* (1968): ethyl; 2,3-dimethyl-6-ethyl. van der Wal *et al.* (1968): 2-methyl-6-isoamyl; 2-methyl-6-(2-methylbutyl); 2,5-dimethyl-3,6-diethyl; 2,6-dimethyl-3,5-diethyl; 2,6-dimethyl-3-isoamyl. van der Wal *et al.* (1971): isopropyl; 2,5-diethyl; 2-methyl-6-(3-methylbutyl); 2,5-dimethyl-3-isobutyl; 2,5-dimethyl-3-(2-methylbutyl); 2,5-dimethyl-3-(3-methylbutyl); 2,6-dimethyl-3-(3-methylbutyl).

While a large number of pyrazines have been identified in food products, critical quantitative information is almost nonexistent. The only published study is that of Müggler-Chavan and Reymond (1967) who employed a peak ratio technique to reveal differences among several varieties of cocoa beans. This was strictly a comparison involving glc peak areas, and actual concentration data were not collected.

In the pyrazine study reported herein, quantitative aspects have been emphasized, especially as they relate to the different sources of cocoa beans, the fermentation process, and roasting practices.

EXPERIMENTAL

Source of Cocoa Beans. Cocoa beans from the major producing countries were supplied by several chocolate manufacturers. Included were beans from Brazil (Bahia), Ghana, Ecuador (Arriba), Mexico (Tabasco), the Dominican Republic (Sanchez), and Samoa.

Pyrazine Analysis. Cocoa beans (30 g) and 20 g of Celite 545 were pulverized 5 min in a Waring Blendor. This mixture

Division of Food Science and Industry, The Pennsylvania State University, University Park, Pennsylvania 16802.
[1] Present address: Department of Food Science and Industries, University of Minnesota, St. Paul, Minnesota 55101.