The relative proportions of citric to malic acid determined in this study differ considerably from those stated by Nelson (1925) and Collins (1960). The differences are attributed to the greater precision and accuracy of the newer methods employed in this study. The use of these newer methods has also led to the elucidation of previously unreported acids in pineapple.

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Carbohydrate and Cyclitol Content of Cannabis

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The carbohydrate and cyclitol content of *Cannabis sativa* grown in the United States (MS-13), Thailand, and Viet Nam was determined *via* silylation and gas chromatographic techniques, and the methods of isolation are described. MS-13 contained the carbohydrates ribitol, fructose, α- and β-D-glucose, and sucrose and the cyclitols (+)-quebrachitol, D(−)-bornesitol, and *myo*-inositol. Only the Thailand sample contained (+)-inositol, whereas only the Viet Nam sample contained erythritol. The carbohydrate–cyclitol content was MS-13 > Thailand > Viet Nam.

In the last decade, those compounds indigenous to *Cannabis sativa* have received much attention and elegant research on the isolation and identification of cannabinol, cannabidiol, the psychotomimetically active Δ^9-tetrahydrocannabinol, and other cannabinoid isomers have been reported (Gaoni and Mechoulam, 1971; Joyce and Curry, 1970). However, there is a paucity of information concerning the classification and amounts of other compounds present in this plant material. Numerous noncannabinoid terpenes have been identified by gas chromatography (gc) and constituted 0.1% of the leaf (Martin *et al.*, 1961; Nigram *et al.*, 1965). Muscarine, choline, and trigonelline have been isolated (Brecht and Salemink, 1969; Salemink *et al.*, 1965) and, more recently, several unknown alkaloids (0.003%) have been reported (Klein *et al.*, 1971). Qualitatively, Adams *et al.* (1940) isolated and identified the cyclitol, quebrachitol, in the steam distillate of an ethanolic extract of Cannabis. In all studies, however, the largest class of compounds to be isolated and identified is the cannabinoids themselves.

It has been suggested that 41% of the phenols found in the mainstream of cigarette smoke derive from the carbohydrate content of the flue-cured tobacco leaf (Bell *et al.*, 1966). In view of the fact that the common usage of Cannabis is *via* the smoking process, it was of interest to determine qualitatively and quantitatively the carbohydrate content of this plant material. Additionally, since cyclitols are polyhydroxylcyclohexanes, dehydration mechanisms can be proposed which would lead to the production of phenols, and knowledge of the cyclitol content would be similarly useful.

The present communication describes the separation techniques and analysis of three samples of Cannabis from different origins and their carbohydrate and cyclitol contents.

EXPERIMENTAL SECTION

A Beckman CC-4 equipped with a flame ionization detector was used as a single column instrument with a Model 3370A Hewlett-Packard electronic integrator. The injection block and detector line were maintained at 260°, detector block was at 350°, and all runs were programmed from 100 to 164° at 2°/min and then from 164 to 252° at 8°/min, with a helium flow of 15 ml/min at a pressure of 80 psi. A 10 ft × ⅛ in. stainless steel column containing 2% OV-17 on Gas Chrom Q (80–100 mesh) was employed.

Standard trimethylsilyl (TMS) sugar solutions of tetra-TMS-L-arabinose, penta-TMS-β-D-fructose, penta-TMS-D-galactose, penta-TMS-α-D-glucose, penta-TMS-β-D-glucose, octa-TMS-lactose, octa-TMS-maltose, penta-TMS-D-mannose, tetra-TMS-C-ribose, penta-TMS-L-sorbose, octa-TMS-sucrose, and tetra-TMS-D-xylose were obtained from Pierce Chemical Co., Rockford, Ill. Free sugars, obtained from Nutritional Biochemicals, Inc., Cleveland, Ohio, were L-fucose, L-glucose, *N*-acetyl-*O*-galactosamine, and *N*-acetyl-*O*-glucosamine; from Calbiochem, Los Angeles, Calif., 3-*O*-methyl-D-glucose and D-galactonolactone; from Mann Research Laboratories, New York, N. Y., (+)-quebrachitol; from Pfanstiehl Laboratories, Inc., Waukegan, Ill., D-fructose, ribitol, and *meso*-erythritol. Supplied from other sources were L-rhamnose, *myo*-inosi-

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Table I. Total Yields of Aqueous Alcohol and Water Extracts of Cannabis Samples[a]

	MS-13	Thailand	Viet Nam
Aqueous alcohol[b] extract	83.2	40.0	8.6
Aqueous alcohol cyclitols	24.1	7.2	2.3
Water extract[c] hydrolyzate	29.2	39.6	41.3
Water extract hydrolyzate cyclitols	7.6	12.7	9.9

[a]Value in g/kg of air-dried plant material. [b]Weight after chloroform and benzene–ether extraction. [c]Weight after cation and anion exchange resin treatment.

tol, D(−)-bornesitol, (+)-quercitol, L-viburnitol, dambonitol, *neo*-inositol, (+)-inositol, and *allo*-inositol, *epi*-inositol, and *talo*-quercitol. All solvents were reagent grade except hexane, which was purified by standard procedures.

Preparation of Standard for Quantitative Analysis. In order to prepare the standards in as similar a manner as possible to the constitution of the unknown samples, the following procedure was used. A stock solution was prepared by weighing out ribitol (0.0050 g), fructose (0.0300 g), D-glucose (0.0500 g), sucrose (0.600 g), (+)-quebrachitol (0.0500 g), and *myo*-inositol (0.0050 g), dissolving them in H_2O, and making up to 50 ml. Of this, 1 ml was concentrated to dryness at 60° under reduced pressure and 1 ml of *N*-trimethylsilylimidazole (TSIM) was added (Schewe, 1971). The mixture was shaken until the syrup completely dissolved (*ca.* 5–10 min). For gas chromatographic (gc) analysis, triplicate injections of 2 μl were made. A second sample was prepared by pipetting 2.00 ml of the stock solution and treating as before. For this sample, triplicate injections of 2.5 μl were made.

Preparation of Additional Standards. Later identification of erythritol, (+)-inositol, and D(−)-bornesitol in the unknowns made it necessary to prepare additional standards, and the limited supply (2 mg) of D(−)-bornesitol required an alteration in procedure. Samples of erythritol (0.0500 g) and (+)-inositol (0.0500 g) were dissolved in water and made up to 50 ml; 2 ml of this solution was evaporated to dryness and the solid was dissolved in 100 μl of TSIM. A sample of D(−)-bornesitol (40 μl of TSIM) was added and the mixture was diluted with pyridine (860 μl). Duplicate samples of 3 μl were injected to obtain the peak area for one sample. For the second sample, duplicate injections of 6 μl were used.

Calculation of Peak Counts for α-D-Glucose and Fructose. The chromatographic peak for α-D-glucose coincided with that of the fifth peak of the fructose anomers (fructose$_5$). Multiple injections of the TMS ethers of water-equilibrated glucose indicated that 57% of the total glucose counts were in the β-D-glucose peak. From this the count for α-D-glucose was calculated and substracted to give the fructose count. Fructose was then determined by totaling the area counts for all the anomers (fructose$_{1-5}$).

Calculation of (+)-Inositol. As the chromatographic peak for (+)-inositol in Thailand extracts coincided with that of α-D-glucose and one of the fructose anomers, the quantitative data were taken from chromatograms of the cyclitols after removal of the reducing sugars. From these data, counts per milligram were calculated and substracted from the counts in the glucose–fructose peak.

Alcohol Extraction Procedure (Davis *et al.*, 1970). Twice-manicured (10 mesh) *Cannabis sativa* (1.00 kg of Ms-13 Male Mexican 1969) and 95% ethanol were allowed to stand in the following order: 7.2 l. for 24, 36, and 65 hr; and 4.5 l. for 10 days. The alcohol was drained and concentrated to 1 l. under vacuum, keeping the temperature below 40°. The concentrate was mixed with 600 ml of water and partitioned first with 900 ml of hexane and then three more times with 180 ml of hexane. The hexane extracts were combined and concentrated to *ca.* 150 ml and stored in a dark bottle at −5° under nitrogen. The total aqueous alcohol phase (1.6 l.) was concentrated to a thick syrup in a vacuum flash evaporator, keeping the temperature below 60°, and the distillate (1.2 l.) was saved for further investigation.

The syrup (106 g, 13.5% moisture) was dissolved in water (600 ml) and extracted four times with 250-ml fractions of chloroform and three times with 250-ml fractions of a benzene–ether mixture (1:1, v/v). The remaining water phase was evaporated again to a syrup (83.2 g, 78.5% yield). Samples of this (35 mg) were silylated with 1 ml of TSIM and analyzed by gc. Quebrachitol was isolated from this syrup by the method of Adams *et al.* (1940) and its presence was confirmed by comparison with a known sample.

Water Extraction Procedure. The plant material (0.789 kg) remaining after four alcohol extractions was twice steeped in distilled water (7.8 l.) which had been boiled and saturated with toluene to prevent growth of microorganisms. After 5 days the water was filtered through Celite and the filtrate was concentrated to syrup in the same manner as the alcohol.

The syrup from both water extracts (290 g, 18.5% moisture) was dissolved in water and extracted six times with chloroform and four times with benzene–ether (1:1, v/v, *vide supra*). The remaining aqueous layer was concentrated to a syrup and dried several times *via* the benzene–ethanol azeotrope.

A sample of syrup (8.54 g) was dissolved in 1 *N* H_2SO_4 (250 ml) and heated in a boiling water bath for 10 hr to hydrolyze the polysaccharides. Barium hydroxide was added carefully to adjust the pH to 6. The solution was filtered and concentrated to a syrup on a vacuum rotary evaporator at temperatures below 60°. A yield of 4.77 g (55.8%) was obtained. Duplicate samples (1.5 g in 10 ml of water) of this hydrolyzate were passed through a column of Rexyn 101 (H^+ form, 25 ml wet volume in a 25-ml buret) and eluted with 850 ml of water. The total eluate was concentrated to a syrup, redissolved (10 ml of water), and passed through a column of Rexyn 203 (OH^- form, 25 ml wet volume in a 25-ml buret). Again 850 ml of water was used to elute the sample. The eluate was concentrated to dryness and the average yield was 0.438 g (29.2%). This product was used for silylation and gc analysis as above.

Thailand and Viet Nam Cannabis. The procedures described for the MS-13 Mexican Cannabis were followed as exactly as possible, except that the amount of starting material was only 10 g. Alterations in the procedure were the neutralization of the hydrolyzates with Rexyn 103 (OH^- form) rather than with barium hydroxide and the use of 15 mg of the resulting syrup for silylation with 1 ml of TSIM for gc analysis.

Removal of Reducing Sugars. The efficiency of removing reducing sugars from cyclitols and other polyhydroxy compounds by treatment with barium hydroxide followed by deionization was established by Riggs and Strong (1967) and the following procedure is an adaptation of that method. Triplicate samples (0.20 g) of the syrup from the aqueous alcohol extract from MS-13 were dissolved in cold saturated barium hydroxide (3 ml) and heated in a boiling water bath for 2 hr. Each of the cooled solutions was then passed successively through columns (7 mm bore) containing Rexyn 101 (H^+ form, 2–3 ml) and Rexyn 201 (OH^- form, 2–3 ml), followed by elution with a minimum of 30 ml of water. After evaporation to dryness on a rotary evaporator, the samples averaged 0.059 g (30% recovery).

A known sample containing (+)-quebrachitol (100 mg), (+)-inositol (10 mg), *myo*-inositol (10 mg), and pinitol (10 mg) was subjected to the same procedure with a near quantitative recovery of 98.6%.

The barium hydroxide–ion exchange treatment was applied to the ethanol extracts and the aqueous extract hydrolyzates from MS-13 Cannabis, Thailand Cannabis, and Viet Nam Cannabis. Table I shows the yields calculated as grams per kilogram of air-dried plant material.

Bromine Oxidation of Aldoses. Since D-mannose was not resolved from one of the anomers of D-fructose under the gc conditions employed, the procedure of Isbell (1963) was used to remove the aldose as mannuronic acid and ascertain if the area observed was for fructose. Portions of the syrup resulting were silylated and analyzed by gc as described.

RESULTS AND DISCUSSION

Since Sweeley and coworkers (1963) reported the gas chromatographic (gc) separation of the trimethylsilyl (TMS) derivatives of carbohydrates, a number of articles have appeared in which these derivatives were used to qualitatively and quantitatively determine the carbohydrate content from various sources using various polar and nonpolar substrates (Davison and Young, 1961; Holtz, 1971; Honig *et al.*, 1971; Laver *et al.*, 1967; Martin and Eib, 1968; Mason and Slover, 1971; Sherman and Goodwin, 1969). We investigated a number of such phases and found that OV-17 (2%) on Gas Chrom Q (80–100 mesh) was the only substance that would resolve the five anomeric forms of fructose (fructose$_{1-5}$) although Curtius *et al.* (1968) reported the separation of compounds with 3% EGS on Chromosorb W 80–100 mesh. Interestingly, although it might be expected that 100–120 mesh would produce better resolution due to increased surface area, the resolution decreased and fructose appeared as one peak. In addition, it was found that any program faster than 2° per minute reduced the resolution, while a slower rate of temperature rise did not improve the separation. Table II compares the retention times of a number of mono- and disaccharide TMS derivatives determined on 2% OV-17 and 5% DEGS. The anomers of L-rhamnose, fructose, and 3-*O*-methyl-D-glucose were separated by OV-17, while DEGS failed to separate those of L-rhamnose and fructose. However, the first anomer of L-rhamnose and D-ribose were not separated by OV-17 but could be on DEGS, and the same thing is to be noted for D-galactose, L-sorbose, and one of the anomers of 3-*O*-methyl-D-glucose. Mannose had the same retention time as fructose on DEGS, while on OV-17 the anomer of fructose$_3$ had the same retention time as did mannose. The disaccharide separation was superior on OV-17 and this separation could be improved by programming at a lower rate than 8° per minute but, since only sucrose was present in the plant extract, the faster program was used. Lactose and sucrose could not be separated on DEGS.

Table II. Relative Retention Times for TMS Derivatives of Carbohydrates[a]

Carbohydrate	OV-17[b]	DEGS[c]
L-Arabinose	0.42	0.48
D-Ribose	0.44	0.52
L-Rhamnose$_1$[d]	0.44	0.49
L-Rhamnose$_2$	0.50	
D-Xylose	0.56	0.59
L-Fucose	0.56	
D-Fructose$_1$	0.64	0.74
D-Fructose$_2$	0.67	
D-Fructose$_3$	0.68	
D-Fructose$_4$	0.78	
D-Fructose$_5$	0.84	
D-Mannose	0.68	0.74
D-Galactose	0.78	0.82
L-Sorbose	0.78	0.79
3-*O*-Methyl-D-glucose$_1$	0.68	
3-*O*-Methyl-D-glucose$_2$	0.78	
α-D-Glucose	0.84	0.85
D-Galactonolactone	0.92	1.27
β-D-Glucose	1.00	1.00
N-Acetylgalactosamine	1.23	1.31
N-Acetylglucosamine	1.24	1.36
Lactose	1.44	1.80
Sucrose	1.45	1.80
Maltose	1.48	1.88

[a] Relative to β-D-glucose. [b] 2% OV-17 on Gas Chrom Q 80–100 mesh, column 10 ft × ⅛ in. stainless steel, temperature 100–164° at 2°/min, 164–252° at 8°/min, He 15 ml/min, head pressure 80 psi. [c] 5% DEGS on Diatoport S 60–80 mesh, column 8 ft × ⅛ in. Cu., temperature 80–220° at 4°/min, He 20 ml/min. [d] Subscripts refer to the various anomers.

Table III. Relative Retention Times of TMS Derivatives of Allitols and Cyclitols

Compound	RRT[a]
Erythritol	0.230
Ribitol	0.479
talo-Quercitol	0.508
proto-Quercitol	0.590
allo-Inositol	0.618
Pinitol	0.646
neo-Inositol	0.664
(+)-Quebrachitol	0.706
Viburnitol	0.738
(−)-Inositol	0.796
Sequoyitol	0.828
epi-Inositol	0.854
Dambonitol	0.878
Bornesitol	0.910
myo-Inositol	1.000

[a] Retention times relative to *myo*-inositol, 2% OV-17 on Gas Chrom Q 80–100 mesh, column 10 ft × ⅛ in. SS, temperature 100–164° at 2°/min, He 15 ml/min, head pressure 80 psi.

Mannose had the same retention time as fructose$_3$ and we were interested in determining if it was present in Cannabis. As a consequence, a synthetic mixture of fructose, mannose, and glucose was oxidized with bromine and the uronic acids were removed by ion exchange resin. The chromatograms were compared before and after oxidation and the change in area for the fructose anomers was noted. The procedure was then applied to the plant carbohydrates and, since no change was observed for the fructose peak areas, it was concluded that mannose was not present.

Since Adams *et al.* (1940) had found quebrachitol in Cannabis, it was reasoned that other cyclitols might be present and a synthetic mixture of the TMS derivatives of 15 allitols and cyclitols was prepared and analyzed by the silylation–gc technique. Excellent separation was obtained and Table III contains the results.

Following this, our attention was focused on the qualitative determination of those carbohydrates and cyclitols present in the plant material extracts. The syrups obtained (see Experimental Section) from the three sources (United States MS-13, Thailand, and Viet Nam) were silylated and the chromatograms were compared with those obtained from the standard solutions previously described. The compounds suspected of being present were then added to the unknown and the analyses were repeated. The unknown was then oxidized and the reducing sugars were removed from the cyclitols, which were silylated and examined by gc. Identification was accomplished by adding known cyclitols to the unknown and repeating the

Table IV. Carbohydrate and Cyclitol Content of the Ethanol Extract of Cannabis Varieties

Class[a]	RRT[b]	Factor[c]	MS-13[d]	Thailand[d]	Viet Nam[d]
Carbohydrates					
Erythritol	0.243	1.89 (0.03)			0.46
Ribitol	0.522	1.24 (0.09)	0.88	0.65	1.60
D-Fructose$_1$	0.655				
D-Fructose$_2$	0.677				
D-Fructose$_3$	0.702	1.00 (0.06)	19.00	5.69	trace
D-Fructose$_4$	0.796				
D-Fructose$_5$	0.851				
α-D-Glucose	0.851	2.50 (0.30)	5.47	1.51	0.02
β-D-Glucose	1.000	2.50 (0.30)	7.19	2.01	0.02
Sucrose	1.430	1.14 (0.04)	20.00	1.21	0.01
Cyclitols					
Quebrachitol	0.758	0.89 (0.04)	4.83	1.18	0.15
(+)-Inositol	0.838	2.02 (0.11)		0.60	
D(−)-Bornesitol	0.956	1.72 (0.06)	2.08	0.87	0.02
myo-Inositol	1.080	12.5 (0.08)	1.67	0.71	0.03

[a]Determined as trimethylsilyl ethers. [b]Retention time relative to β-D-glucose on 10 ft × 1/8 in. stainless steel column with 2% OV-17 on Gas Chrom Q 80–100 mesh, He flow 15 ml/min, temperature programmed 100–164° at 2°/min, 164–252° at 8°/min. [c]Milligrams of compound per count × 10^7 (standard deviation). [d]Values in g/kg of air-dried plant material.

analysis. In this way MS-13 was found to contain ribitol, fructose, α- and β-D-glucose, sucrose, quebrachitol, D(−)-bornesitol, and *myo*-inositol. For quantitative determination, a stock solution of these compounds was made up and the gc area count was determined for each compound. Knowing then the milligrams per count, this factor was multiplied by the counts determined for each compound obtained from the alcohol extract and the water hydrolyzate.

In the case of the five fructose anomer peaks, it was noted that the areas for individual anomers changed with time, although the total count was fairly constant, particularly with TSIM as the silylating agent. This is reflected in the multiplying factor of 1.00 ± 0.06, which is comparable to the factors for the other compounds (Tables IV and V).

Table V. Carbohydrate and Cyclitol Content of Cannabis Water Extract Hydrolyzates

Class[a]	RRT[b]	Factor[c]	MS-13[d]	Thailand[d]	Viet Nam[d]
Carbohydrates					
Erythritol	0.243	1.89 (0.03)			
Ribitol	0.522	1.24 (0.09)	2.24		
D-Fructose$_1$	0.655				
D-Fructose$_2$	0.677				
D-Fructose$_3$	0.702	1.00 ± (0.06)	7.62	3.13	
D-Fructose$_4$	0.796				
D-Fructose$_5$	0.851				
α-D-Glucose	0.851	2.5 ± (0.3)	4.42	1.36	0.18
β-D-Glucose	1.000	2.5 ± (0.3)	5.84	1.78	0.24
Sucrose	1.43	1.4 ± (0.04)			
Cyclitols					
Quebrachitol	0.758	0.89 (0.04)	1.97	0.28	0.17
(+)-Inositol	0.838	2.02 (0.11)	0.32	0.20	0.03
D(−)-Bornesitol	0.956	1.72 (0.06)	0.87	0.21	0.07
myo-Inositol	1.08	1.25 (0.08)	4.70	1.04	0.20

[a]Determined as trimethylsilyl ethers. [b]Retention time relative to β-D-glucose on 10 ft × 1/8 in. stainless steel column with 2% OV-17 on Gas Chrom Q 80–100 mesh, He flow 25 ml/min, temperature programmed 100–164° at 2°/min, 164–252° at 8°/min. [c]Milligrams of compound per count × 10^7 (standard deviation). [d]Values in g/kg of air-dried plant material.

As shown in Table IV, MS-13 had the highest carbohydrate and cyclitol content. Where MS-13 had a fructose-sucrose ratio of approximately 1, that of the Thailand sample was 4.5. In addition to gross differences in weight content, only the Thailand plant material contained (+)-inositol, while the Viet Nam sample was the only one to contain erythritol.

The water extract of the plant material contained the alcohol-insoluble polysaccharides which were hydrolyzed and analyzed. As shown in Table V, the MS-13 sample again had the highest total compound concentration and the ribitol and *myo*-inositol content of MS-13 was higher in the water extract than in the alcohol extract.

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