Gas Chromatography of Carbohydrates

THEODORE CAYLE, FRED VIEBROCK, and **JOAN SCHIAFFINO,** Wallerstein Company, Division of Baxter Laboratories, Wallerstein Square, Staten Island, New York 10303

ABSTRACT

Conditions have been established for quantitatively determining the major carbohydrate components in starch syrups without the formation of the anomeric forms associated with the ordinary trimethylsilyl (TMS) derivatives. The carbohydrates, including an internal standard of ribose, are reduced with borohydride. Excess reagent is destroyed by HF, and the borate is removed by treatment with a mixed-bed ion-exchange resin, followed by distillation as methyl borate. The relatively insoluble reduced carbohydrates are dissolved in a mixture of dimethylsulfoxide and pyridine, and TMS derivatives are prepared with the conventional reagents, hexamethyldisilazane and trimethylchlorosilane. By controlling the relative concentration of reagents, intermediates are obtained within a single liquid phase, providing for easy handling of the samples. Reducing the total number of peaks obtainable from a mixture of carbohydrates allows for more precise quantitative evaluation of the data. This also allows for estimation of components having retention times similar to one of the anomeric pairs present as unreduced TMS derivative.

Since the use of gas-liquid chromatography for separation of carbohydrates was first reported (1), many modifications and improvements in technique have been published. Sweeley *et al.* (2) described a simple and rapid method for preparing trimethylsilyl (TMS) derivatives of sugars and related substances, and also discussed some results with reduced TMS carbohydrates. Richey *et al.* (3) called attention to variability in the quantitative determination of carbohydrates caused by poor solubility of some of the sugars in pyridine, and the requirement to sum the area of several peaks for a single sugar because of the formation of anomers. This latter point has also been found to contribute unnecessarily to the complexity of a chromatogram when a number of overlapping peaks are obtained with carbohydrate mixtures.

Sawardeker *et al.* (4) converted monosaccharides to their alditols to prevent anomerization and ring isomerization. These were then chromatographed as acetates.

Brobst and Lott (5) contributed to the technique for chromatographing corn syrups by obviating the requirement for drying samples, using an excess of hexamethyldisilazane and trifluoroacetic acid. This allowed for direct trimethylsilylation of commercial syrups. However, their procedure resulted in the multiple peaks per component described above, required daily calibration of the chromatograph, and did not provide for separation of such sugars as sucrose from typical corn syrup components without resort to a change of columns.

When we attempted to make use of information available in the literature to quantitatively determine the carbohydrates present in corn syrups, using the reduced form of the sugars as a means of prohibiting anomerization and ring isomerization, we found that results were unreliable and unreproducible. Among the difficulties encountered were poor solubility of the reduced sugars, or development of multiphase systems, in solvents previously reported to be satisfactory for the unreduced forms. In addition, borate complexing inter-

fered with the formation of TMS derivatives and introduced considerable variability in the chromatographic response.

This paper describes a procedure which can be used to determine quantitatively the major syrup components; the rapid forming TMS derivatives and an internal standard are used. Single peaks are obtained for each component.

MATERIALS AND METHODS

Apparatus

The gas chromatograph used was an F&M 810R-19, with dual columns, dual flame ionization detector, a 201-B Disc integrator, and a Model 50 automatic attenuator (optional).

The two columns were 2 ft., 0.25 in. o.d. stainless steel, packed with 3% silicone rubber SE-52 on 80- to 100-mesh Diataport S (F&M Scientific Corp., Avondale, Pa.). Column ends were plugged with glass wool and new columns were conditioned at 300°C. overnight with carrier gas.

Conditions for operation: Injection port, 300°C.; detector, 300°C.; oven, 140°–275°C. (programmed at 6°/min); post-injection interval, 4 min.; hold at 275°C. until maltotriose eluted (approximately 25 min.); range, 10^2; minimum attenuation, 2 for monose and diose, 1 for triose; gas pressures: helium 25 lb., 80 ml./min., air 35 lb., 520 ml./min., and hydrogen 20 lb., 50 ml./min.; chart speed, 4 min./in. during post-injection period, 20 sec./in. for monose and diose, 4 min./in. for triose.

A Rinco flash evaporator proved very useful where indicated in the procedure.

Procedure

Place a quantity of corn syrup equivalent to 260 mg. of syrup solids in a polyethylene bottle and add 2.0 ml. of a 2.0% aqueous solution of ribose (40 mg.) as an internal standard. Add 150 mg. of sodium borohydride[1] as a 10% solution (equivalent to 50% w./w. of the total carbohydrate), and allow reaction to proceed for 1 hr. at room temperature with gentle stirring. Midway during this period, wash down sides of bottle with 5 ml. distilled water to make certain that all sugar has reacted. Add 1.3 ml. of a 25% solution of hydrofluoric acid dropwise from a plastic pipet. Dilute with distilled water to a concentration of 10 mg. of carbohydrate per ml. Add 6 g. of Amberlite MB-3[2] ion-exchange resin (equivalent to 40 times the weight of $NaBH_4$ used). Stir for 2 hr. at room temperature, then centrifuge. Transfer 3 ml. of the supernatant material to a 100-ml. pear-shaped flask, add 20 ml. anhydrous methanol, and evaporate to dryness under vacuum in the Rinco evaporator. Gentle warming may be used. Extract the residue six times with 20-ml. portions of anhydrous methanol, and evaporate to dryness (this normally does not take longer than 30 min.). Dissolve the residue in 1 ml. dimethylsulfoxide[3]. Add 2.0 ml. anhydrous pyridine and several crystals of anhydrous Na_2SO_4. Add 0.4 ml. hexamethyldisilizane[4] and 0.6 ml. trimethyl-

[1] Sodium borohydride, Metal Hydrides, Inc.
[2] Amberlite MB-3 mixed-bed ion-exchange resin, Mallinkrodt Chem. Works.
[3] Dimethylsulfoxide, Matheson, Coleman, and Bell.
[4] Hexamethyldisilizane, Peninsular Chemical Research, Inc.

chlorosilane[5], shake, and warm slightly to hasten the reaction. After 15 min. the mixture is ready for analysis. Inject 0.5 μl.

Calibration and Calculations

Calibration and calculations with the internal standard were made according to Sawardeker and Sloneker (6). D-Ribose was chosen as the internal standard because of its desirable retention time with respect to the sugars under study. Linear calibration curves were obtained by chromatographing TMS alditol derivatives of glucose, maltose, and maltotriose, to which a constant amount of ribose was added prior to reduction, and by plotting the ratio of the area of each TMS alditol peak to that of the standard, against the ratio of the weight of the aldose to that of the D-ribose in the mixtures. The detector response (K) values were then determined from the slope of the curves.

$$K = \frac{\text{peak area of TMS alditol/peak area of TMS ribitol}}{\text{wt. of aldose/wt. of D-ribose}}$$

$$\text{Wt. of unknown aldose} = \frac{\text{peak area of unknown} \times \text{wt. of ribose}}{\text{peak area of TMS ribitol} \times K}$$

The K values for the TMS alditols of glucose, maltose, and maltotriose were 0.866, 0.511, and 0.384 respectively.

RESULTS

The limit of detector response to the TMS alditols was determined in order to establish the optimum concentration of syrup components to be injected. Although the flame ionization detector responds to the number of molecules passing through per unit time, we have found that peak size is influenced by the volume in which the TMS derivative is injected. The response *vs.* quantity injected as a function of injection volume was established for TMS glucitol (sorbitol) and TMS maltitol. As can be seen from Fig. 1,

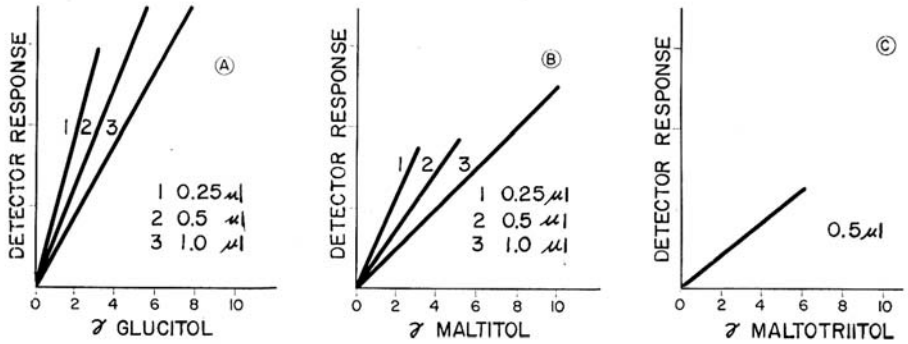

Fig. 1. Relation of detector response to concentration of sugar as a function of injection volume.

the lower the injection volume, the more sensitive is the response of the detector to a given weight of derivative. Curve C of Fig. 1 shows the detector response to increasing levels of maltotriose at a single injection volume.

[5]Trimethylchlorosilane (SC-3001), General Electric Co.

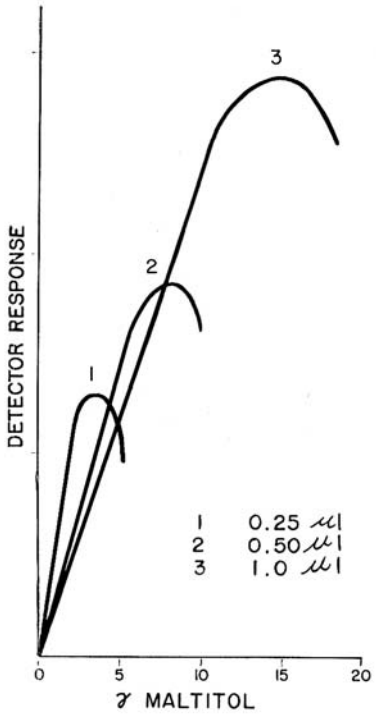

Fig. 2. Relation of detector response to concentration of TMS maltitol as a function of injection volume.

Figure 2 shows the detector response to increasing weights of TMS maltitol in 0.25-, 0.5-, and 1.0-µl. injections. The data indicate that the detector no longer responds in a linear manner when more than 2.5 γ in 0.25 µl., 5.0 γ in 0.5 µl., or 10 γ in 1.0 µl. is injected. The same general relationship held true for the other TMS alditols investigated.

As a compromise with respect to maximum detector sensitivity and reasonable working concentrations of the components under investigation, a 0.5 µl. injection was selected. Under the method outlined above, 0.5 µl. will contain 3.75 γ total carbohydrate, an amount well within the maximum limits of the detector.

The chromatograms in Fig. 3 show a comparison between the unreduced and reduced TMS derivatives of a mixture composed of equal weights of glucose, maltose, and maltotriose. The chromatogram of the unreduced sugars shows the typical multipeak response due to the formation of the alpha and beta anomers of glucose and maltose. The TMS alditols chromatograph as single peaks. Table I shows the recoveries obtained and the expected deviation.

The chromatograms in Fig. 4 show a comparison between TMS derivatives of an unreduced and reduced commercial 65 DE corn syrup. Again, the lack of complexity of the TMS alditol chromatogram, and the ease with

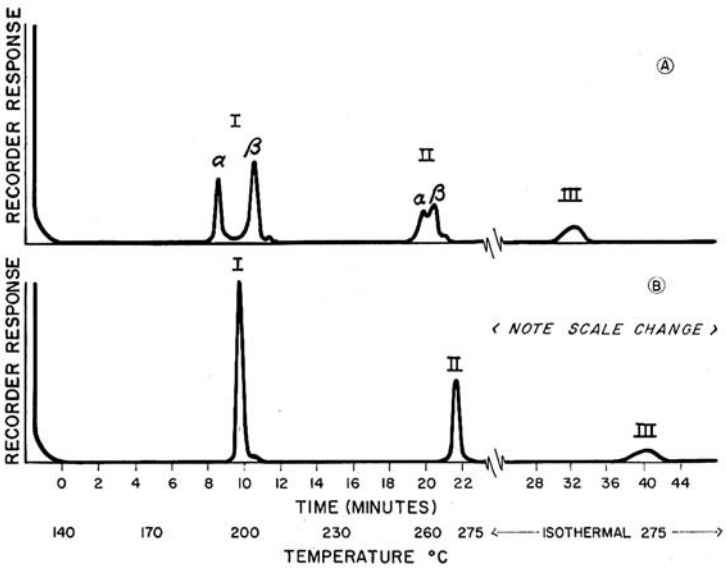

Fig. 3. Gas-liquid chromatograms of TMS derivatives of mixture of equal weights of glucose (I), maltose (II), and maltotriose (III). A, unreduced; B, reduced.

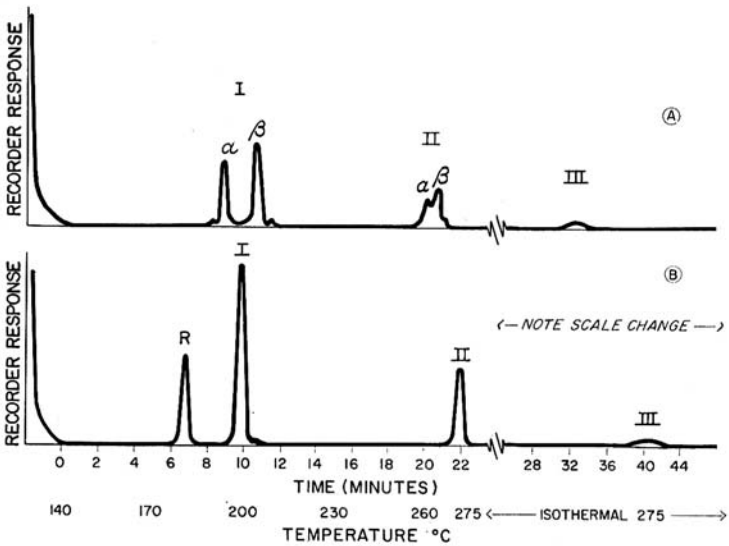

Fig. 4. Gas-liquid chromatograms of TMS derivatives of 65 DE corn syrup. Glucose (I), maltose (II), maltotriose (III). A, unreduced; B, reduced with internal standard (R).

which the areas under the single peaks can be quantitatively evaluated, are apparent. The table below shows the analysis of the reduced syrup.

To establish if fructose and sucrose could be determined in the presence of glucose and maltose under these conditions, a mixture of these sugars

Component	Concentration %
Glucose	33.7 ± 0.8
Maltose $\cdot H_2O$	30.4 ± 0.3
Maltotriose	11.1 ± 0.6

Component	Applied mg.	Recovered mg.	Recovered %
Glucose	50	49.8[a]	99.6
Fructose	50	49.8[a]	99.6
Sucrose	50	51.3	102.6
Maltose $\cdot H_2O$	50	50.4	100.8

[a]Total quantity of sugar represented by glucitol peak divided by 2.

TABLE I
RECOVERY OF SUGARS WITH GAS-LIQUID CHROMATOGRAPHY

GLUCOSE			MALTOSE			MALTOTRIOSE		
Theoretical	Found	Deviation	Theoretical	Found	Deviation	Theoretical	Found	Deviation
mg.	mg.	%	mg.	mg.	%	mg.	mg.	%
200	206.	+3.0	200	198.	−1.0	100	108	+8.0
160	156.	−2.5	160	157.	−1.9	100	91.0	−9.0
120	123.	+2.5	120	128.	+6.7	80	76.0	−5.0
80	78.0	−2.5	80	77.9	−2.6	80	77.0	−3.8
40	39.9	−0.3	40	39.8	−0.5	60	61.7	+2.8
		±2.2%			±2.5%	40	38.1	−4.7
						40	36.9	−7.8
						57.5	56.7	−1.4
								±5.3%

was prepared and analyzed. Chromatogram A of Fig. 5 shows the tracing of the unreduced mixture. As previously reported by Brobst and Lott (5), TMS sucrose overlaps the TMS alpha-maltose peak on a 2-ft. SE-52 column. However, as seen in tracing B, Fig. 5, TMS sucrose clearly separates from the single TMS maltitol peak after the mixture is reduced. Fructose, on the other hand, separates from alpha- and beta-glucose if unreduced, and is converted to products with a retention time identical to that of glucitol by reduction. To determine the level of fructose present when reduction is employed, a glucose value with glucose oxidase can be subtracted from the total apparent glucitol and the fructose estimated by difference.

The table below shows the recovery of glucose, maltose, fructose, and sucrose from a known mixture of these sugars. In this illustration, the recovery of glucose and fructose was calculated by determining the total quantity of sugar represented by the glucitol peak and dividing this value by 2 (equal weights of glucose and fructose were applied). The K value found for TMS sucrose was 0.568.

DISCUSSION

The trimethylsilylation of alditols after reduction with borohydride is not possible without first removing the borate ions that are formed. The use of a mixed-bed ion-exchange resin as suggested by Sweeley *et al.* (2), after excess $NaBH_4$ was decomposed by acidification with HCl, was found to be

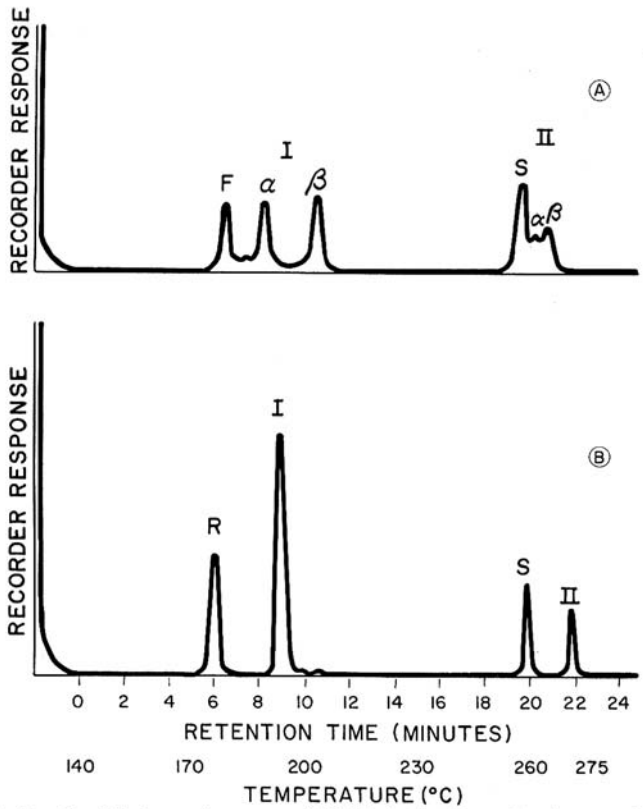

Fig. 5. Gas-liquid chromatograms of TMS derivatives of mixture of glucose (I), fructose (F), sucrose (S), and maltose (II). A, unreduced; B, reduced with internal standard (R).

too inefficient. The distillation of residual borate as methyl borate, as recommended by Zill *et al.* (7), after pretreatment with the mixed-bed resin, allowed for complete removal of borate, provided acidification was carried out with HF rather than HCl.

Maltitol and maltotriitol are less soluble in pyridine than glucitol. Dimethylsulfoxide, which is immiscible with the silylation reagents, readily dissolves the alditols. A mixture of one part dimethylsulfoxide and two parts pyridine provides for complete solution of the reagents and the derivatives.

The derivatives are stable for several hours when kept tightly covered to exclude moisture. Anhydrous Na_2SO_4 is added as a dehydrating agent, and to avoid moisture pick-up during the warming of the reaction, this step is best carried out in a heating mantle filled with lead shot or sand, rather than a water bath.

The flame detector rings are cleaned daily to remove the film of SiO_2 which accumulates. A charge of hexamethyldisilizane is also injected into the column daily and purging is allowed to proceed at 275°C. until a steady base line is obtained. This preliminary treatment reduces the potential number of spurious peaks.

TABLE II
RETENTION TIMES OF SOME TMS CARBOHYDRATES

UNREDUCED		REDUCED	
Component	Retention Time[a]	Component	Retention Time[b]
Alpha-glucose	1.00	Ribitol	0.62
Beta-glucose	1.20	Glucitol	1.00
Fructose	0.92	Sucrose	1.99
Sucrose	2.24	Maltitol	2.23
Alpha-maltose	2.30	Maltotriitol	4.08
Beta-maltose	2.35		
Maltotriose	3.74		

[a]Relative to alpha-glucose.
[b]Relative to glucitol.

Table II shows the relative retention times of the TMS alditols compared to the unreduced TMS derivatives. The more distinct separation of the reduced derivatives may enable us to shorten the length of the columns, thereby reducing the length of time required for each analysis. The complete separation of sucrose from maltitol also permits the use of sucrose as a second internal standard.

Literature Cited

1. MCINNES, A. G., BALL, D. H., COOPER, F. P., and BISHOP, C. T. Separation of carbohydrate derivatives by gas-liquid chromatography. J. Chromatog. 1: 556–557 (1958).
2. SWEELEY, C. C., BENTLEY, R., MAKITA, M., and WELLS, W. W. Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. J. Am. Chem. Soc. 85: 2497–2507 (1963).
3. RICHEY, J. M., RICHEY, H. G., and SCHRAER, R. Quantitative analysis of carbohydrates using gas-liquid chromatography. Anal. Biochem. 9: 272–280 (1964).
4. SAWARDEKER, J. S., SLONEKER, J. H., and JEANES, A. Quantitative determination of monosaccharides as their alditol acetates by gas-liquid chromatography. Anal. Chem. 37: 1602–1604 (1965).
5. BROBST, K. M., and LOTT, C. E., JR. Determination of some components in corn syrup by gas-liquid chromatography of the trimethylsilyl derivatives. Cereal Chem. 43: 35–43 (1966).
6. SAWARDEKER, J. S., and SLONEKER, J. H. Quantitative determination of monosaccharides by gas-liquid chromatography. Anal. Chem. 37: 945–947 (1965).
7. ZILL, L. P., KHYM, J. X., and CHENIAE, G. M. Further studies on the separation of the borate complexes of sugars and related compounds by ion-exchange chromatography. J. Am. Chem. Soc. 75: 1339–1342 (1953).

[Received May 4, 1967. Accepted December 10, 1967]