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GLASS CAPILLARY OR FUSED-SILICA GAS CHROMATOGRAPHY—MASS SPECTROMETRY OF SEVERAL MONOSACCHARIDES AND RELATED SUGARS: IMPROVED RESOLUTION

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SUMMARY

The gas chromatographic separation of several monosaccharides and related sugars derivatized by methoxylation and trimethylsilylation reactions was optimized with glass capillary (SP-2250) and fused silica (SP-2100) columns. Individual sugars included aldoses, ketoses, polyols, acidic forms and N-acetylated amino sugars. Peaks were detected by selected ion monitoring (SIM). The fused silica column gave complete resolution of all peaks (two per hexose and one per hexitol) arising from glucose, galactose, mannose, fructose, sorbitol, mannitol and dulcitol. The resolution of these sugars with the glass capillary column was not as good, but full differentiation was possible on the basis of SIM. Because the fused silica column gave a better resolution of 33 sugars tested and was more easily installed than the glass capillary column, it was utilized for quantitative analysis. A deuterated algal sugar mixture used for quantitation by isotope dilution was found to contain glucose, galactose, mannose, xylose, arabinose, ribose and rhamnose. Full recoveries were obtained of various amounts of glucose, galactose, mannose, fructose and xylose added to human serum.

INTRODUCTION

The International Federation of Clinical Chemistry has recommended the definitive-reference method system for achieving the ultimate in accuracy for reference materials in clinical chemistry [1]. Thus, in order to assign exact glucose values to human sera for evaluating the accuracy of a wide variety of routine glucose methods used in clinical laboratories, it was necessary to first set up a gas chromatographic—mass spectrometric (GC—MS) method capable not only of differentiating glucose but also other monosaccharides and related sugars.

Cohen et al. [2] have proposed two candidate definitive isotope dilution MS methods for serum glucose by converting glucose to 1,2:5,6 di-O-iso-

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propylidine-D-glucofuranose or to D-glucofuranose cyclic 1,2:3,5-bis(butylborate) following intensive preparation and purification steps designed expressly for glucose determination.

Jankowski and Gaudin [3] identified several sugars by MS following trimethylsilylation (TMS) of 5-mg amounts under rigorously controlled silylation conditions and GC on a 91-cm 3% SE-30 packed glass column. In this laboratory, when their procedure was applied to lower glucose concentrations normally encountered in serum, the glucose derivatives gave two peaks with relative intensities of 37% and 63% representing normal proportions of α- and β-anomers instead of the reported values of 3% and 97% [3].

The derivatization method of Laine and Sweeley [4], consisting of methyloximation (MO) followed by TMS avoids the variation in the ratios of α- and β-anomers of the pyranose and furanose forms that yield the same ratios of *syn*- and *anti*-isomers from both α- and β-anomers [5].

Bjorkhem et al. [5] have chromatographed MO-TMS glucose derivatives on a packed 3% SE-30 column but reported no data on the resolution of other sugars such as galactose, mannose and fructose that may be present in human serum. A 50-m glass capillary column coated with SE-30 has been used by Eldjarn et al. [6] for MO-TMS glucose derivatives but they gave no account of the resolution of other sugars. Using a similar column but a somewhat more complex procedure for MO-TMS derivatization, Zegota [7] demonstrated a satisfactory separation of glucose, galactose and fructose but did not report on mannose. According to data obtained by Størset et al. [8] for a 25-m capillary column coated with SE-30, the major peak of mannose had a retention time very close to those of the second peak of fructose and the major peak of galactose; consequently, it appears that even with a 50-m column under the conditions utilized by Zegota [7] there would be insufficient time for a well resolved mannose major peak to fit between the peaks for these two other sugars.

The primary objective of this study was to improve the GC resolution of glucose, galactose, mannose and fructose and a variety of monosaccharides and related sugars derivatized according to the MO-TMS procedure of Laine and Sweeley [4], in conjunction with selected ion monitoring (SIM). The applicability of a high-resolution GC procedure to quantitative measurements of glucose and a few other sugars by isotope dilution with a deuterated algal sugar mixture was also evaluated.

EXPERIMENTAL

Reagents

All of the sugars were obtained from commercial sources. Other reagents were obtained as follows: methoxylamine hydrochloride from Eastman Kodak (Rochester, NY, U.S.A.), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Pierce (Rockford, IL, U.S.A.) and ACS certified pyridine from Fisher Scientific (Fair Lawn, NJ, U.S.A.). The deuterated algal sugar mixture solution of unspecified composition was obtained from Merck Sharp & Dohme Canada (Montreal, Canada).

Sample preparation

The method of derivatization was essentially that of Laine and Sweeley [4] as described by Bjorkhem et al. [5]: a 100-μl volume of serum or of 0.05% benzoic acid containing one or several sugars ($5 \cdot 10^{-3}$ mol/l) was added to 2-ml screw-top vials with Teflon-lined caps and made up to a final volume of 400 μl with 0.05% benzoic acid. After lyophilization, 500 μl of a solution of methoxylamine hydrochloride (1%, w/v) in dry pyridine were added and the sealed vials were heated for 2 h at 80°C with occasional mixing. Subsequently, 150 μl of BSTFA were added and allowed to react for 15 min at 80°C with occasional mixing.

Gas chromatography—mass spectrometry

The derivatized solutions were analysed with a Finnigan GCMS Model 4000 instrument consisting of a quadrupole spectrometer, a Model 9610 gas chromatograph and a 6110 data system. The ionizer was normally set at 70 eV, except for quantitation at 40 eV, and was operated at 250°C. The temperature of the transfer oven was also set at 250°C. The glass capillary column (30 m × 0.245 mm I.D.), coated with SP-2250 (methylphenylsilicone) was supplied by J & W Scientific (Rancho Cordova, CA, U.S.A.). The fused silica column (50 m × 0.2 mm I.D.), deactivated with Carbowax 20M and coated with SP-2100 (methylsilicone), was supplied by Hewlett-Packard (Canada) (Mississauga, Canada). The columns were adjusted to a height of about 10 mm from the bottom of the glass insert tube into the injector. The other end of the fused silica column was inserted directly into the GC—MS transfer line at a distance of about 1 mm from the ion source block. The temperature of the injector was set at 250°C.

With the glass capillary column, a volume of 1 μl was injected in the split mode (20:1) at a column flow-rate of helium of 2 ml/min, a sweep of 10 ml/min and an oven temperature of 75°C; after 30 sec the oven temperature was programmed to rise to 150°C at 10°C/min, where it was held for 23 min, and finally at full capacity to 180°C.

With the fused silica column, a volume of 3 μl was injected in the split mode (10:1) with a column flow-rate of helium of 1 ml/min, a sweep of 10 ml/min and an oven temperature of 180°C.

For the analysis of pentoses and pentitols, additional experiments were done with the fused silica column at an oven temperature of 140°C and with the glass capillary column utilizing a programme where the oven temperature was raised from 75°C to 100°C at 2°C/min and from 100°C to 180°C at 10°C/min.

RESULTS AND DISCUSSION

Selected EI ion pattern of sugars and derivatives

Similarities and differences in the ion patterns of various sugars derivatized by MO-TMS reactions and chromatographed on the SP-2100 fused silica column are summarized in Table I. A similar ion pattern (not shown) with differences in elution order was obtained with the SP-2250 glass capillary column. Characteristic ions in Table I were selected on the basis of the mass

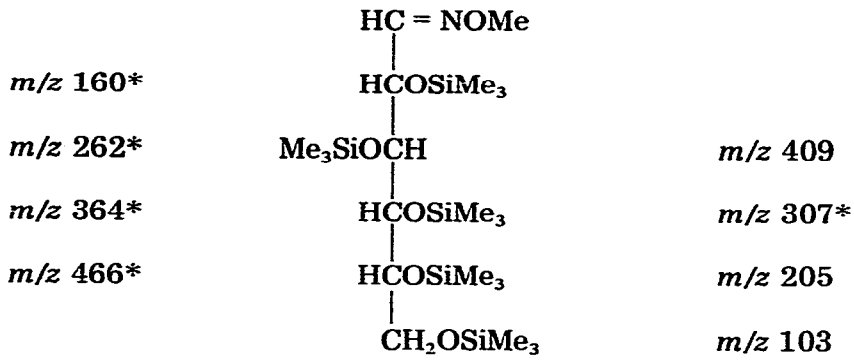
TABLE I

SELECTED ION PATTERN OF SUGARS DERIVATIZED WITH MO-TMS AND CHROMATOGRAPHED ON SP-2100 FUSED SILICA COLUMN

Sugar*	Selected ions (numerically decreasing intensities)								
	160	173	229	231	262	277	307	319	Various
Erythrol (A, 1)				2		4	1		293/3 320/5
Xylose (B, 2)	2	5	5	5	7	4	1		232/3
Lyxose (C, 2)	2	5		5	7	3	1		233/4
Arabinose (D, 1)	2	5		6	6	3	1		233/3
Ribulose (E, 2)		1		4					232/3 263/2 333/6 364/5
Xylulose (F, 1)		1		5					232/4 263/2 333/6 364/3
Ribose (G, 1)	2	5		5	5	3	1		233/3
Rhamnose (H, 2)	1	6		4		2			233/3 321/5
Fucose (I, 2)	1	6		3		2			233/4 321/5
Xylitol (J, 1)			5			4	1	2	243/3 332/6 422/7
Arabitol (K, 1)			5			4	1	2	243/3 332/6
Adonitol (L, 1)			5			4	2	1	243/3 332/6 422/7
Deoxyglucose (M, 1)							4	3	174/1 244/6 276/2 288/5
Gluconic acid (N, 2)			2	5				1	174/3 243/4 333/5 361/7
Glucuronic acid (O, 1)	1								174/2 244/4 274/3 288/4
Tagatose (P, 3)		2			4	2	1		364/4
Sorbose (Q, 2)		2			2	2	1		364/5
Fructose (R, 2)		3		4		2	1		263/4 364/4
Gluconic acid (N, 1)				5					244/1 305/4 332/2 334/3
Allose (S, 2)	2	5	3	4	5	5	5	1	233/5
Mannose (T, 2)	1	5	3	4	8	8	5	2	233/5 364/10
Talose (U, 3)	1	7	3	4	8	8	6	2	233/5
Galactose (V, 2)	1	6	3	4	6	6	5	2	233/6
Glucose (W, 2)	1	7	3	4	7	7	5	2	233/5 364/7
Sedoheptulose (X, 1)			5	4					243/2 317/3 333/1 375/5
Glucuronic acid (O, 2)	1								305/3 333/2 364/4 423/5
Mannitol (1, 1)			3	4		5	2	1	331/7 345/6 421/8
Sorbitol (2, 1)			3	4		5	2	1	331/6 345/6 421/8
Dulcitol (3, 1)			4	3		5	2	1	331/5 345/7 421/7
N-Acetyl-									
glucosamine (4, 2)		1	7	4	4			2	274/4 333/3
mannosamine (5, 2)		1	4	3	5			2	274/5
galactosamine (6, 1)		1	6	3	4			2	274/4 333/6
Myoinositol (7, 1)									265/3 305/1 318/2 432/4
Mannoheptulose (8, 2)		4	3		2		5	1	
Glucoheptulose (9, 2)	1			6	6		2	3	259/4 331/4

*In order of t_R of major or single peak. First character within parentheses identifies peaks in chromatograms and is followed by the number of peaks for each sugar. Gluconic and glucuronic acid each gave two distinct ion patterns.

spectrum of the peaks from individual sugars, and the relative intensities at each characteristic *m/z* were evaluated from each sugar. Laine and Sweeley [4] have explained the origin of some ions from glucose according to the following scheme, where Me represents methyl groups:



The ions at *m/z* 160, 262, 364, and 307 marked with an asterisk (see above scheme) are listed in Table I. As reported by Laine and Sweeley [4], ions at *m/z* 409 were not detected, but in contrast no ion was observed at *m/z* 466, possibly because we injected smaller amounts. Ions at *m/z* 205 and 103 were deleted from Table I because they were considered to be non-specific. According to Laine and Sweeley [4], the presence of other ions can be explained as follows:

1. Ions at *m/z* 409 — 90 (Me_3SiOH) = *m/z* 319.
2. Ions at *m/z* 409 — 180 (2 Me_3SiOH) = *m/z* 229.

In addition, *m/z* 277 could be explained as for TMS derivatives of hexitols [9] by expulsion of CH_2O from the ions of *m/z* 307. The intensities at *m/z* 173 produced by glucose and other aldoses, although weak, were listed because of the importance of *m/z* 173 for identifying ketoses and N-acetylated hexosamines. Similarly, a relatively low intensity at *m/z* 231 for aldoses, ketopentoses and the first peak of fructose (the second showing none) were listed because the intensity at *m/z* 231 for fucose, dulcitol, N-acetylmannosamine and N-acetylgalactosamine ranked third in decreasing order among the ions selected for these sugars. Although intensities at *m/z* 233 were not important for the identification of all aldoses, ketopentoses and 6-deoxysugars, their intensities were listed because intensities at *m/z* 233 were not negligible (ranking third) for xylose, arabinose, ribose and rhamnose. A thorough explanation of the origin of all specific masses for the various sugars, including those at *m/z* 173, 231 and 233, is outside the scope of this paper.

Laine and Sweeley [10] have discussed the mass spectra of the MO-TMS derivatives of xylose, ribose, fucose, fructose and glucoheptulose. The fragmentation pattern of MO-TMS derivatives of N-acetylated hexosamines has been covered by Orme et al. [11]. Discussions of mass spectra for TMS derivatives for pentitols and hexitols by Petersson [9] and for inositol by Sherman et al. [12] also apply to the derivatives in the present study.

In general, the spectral similarities for stereoisomers within each of various groups such as aldopentoses, ketopentoses, pentitols, 6-deoxyhexoses, hexitols and N-acetylated hexosamines demonstrate that separation of the stereoisomers is a prerequisite for their identification.

Glass capillary column gas chromatography

In our experience, it was not possible to separate galactose and mannose

with a glass capillary column coated with SE-30, and none of the authors reviewed [5—8] using this type of column with MO-TMS derivatives have reported the separation of these two sugars together with glucose. Fig. 1 (central portion) demonstrates a satisfactory separation of these three sugars with a glass capillary column coated with a more polar phase (SP-2250). Each of these sugars in a mixture produced two peaks (representing *syn* and *anti* forms) at *m/z* 319 and 160; these peaks were well resolved, except for the minor peak of mannose, which shouldered the major peak of galactose but not to the extent that would produce interference in their peak height measurements. Tagatose and fructose contained in the same mixture gave no intensity at *m/z* 319 and 160 and were well resolved at *m/z* 307. Injection of another mixture containing allose, sorbose and talose showed that the minor peak of talose eluted at the same time as the major peak of glucose and the major peak of talose would interfere with peak-area and not peak-height measurements of the major peak of galactose; in practice, talose would not be expected to occur naturally in biological samples. Allose would not present any problem in the resolution of the other aldoses, but it produced some intensities coincident with the first fructose peak at *m/z* 307. The first and second peak of sorbose coincided with the second peak of tagatose and fructose, respectively. A separate injection of a mixture of hexitols showed that these would interfere with some of the aldose peaks at *m/z* 319 but not 160; the three hexitols were completely resolved at *m/z* 307.

Deoxy-D-glucose gave two adjacent peaks that eluted before the hexoses, and was preceded (in the first segment of Fig. 1) by the 6-deoxysugars: fucose

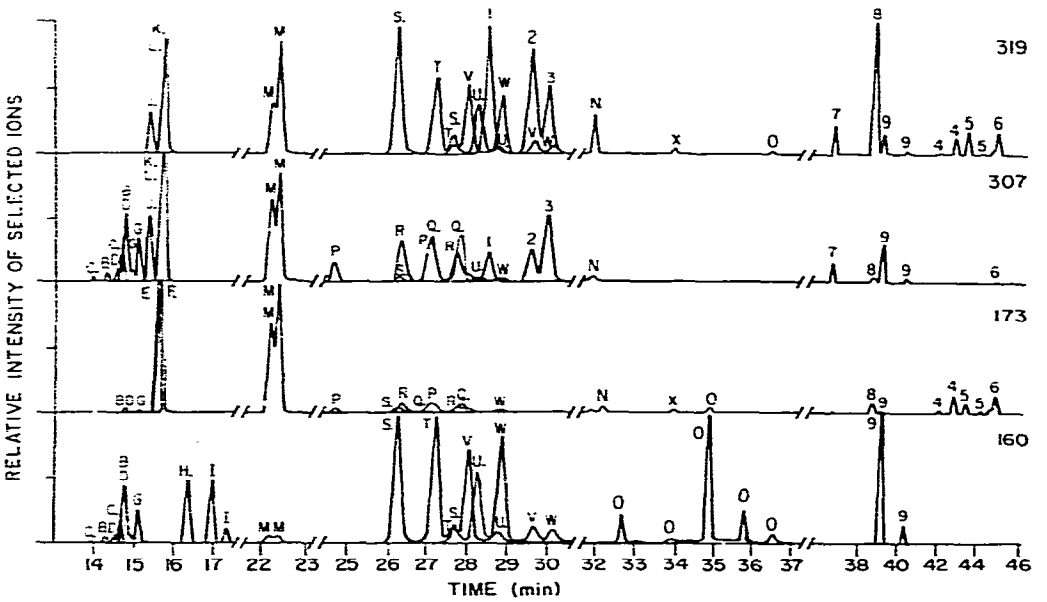


Fig. 1. Glass capillary (SP-2250) gas chromatography of various sugars derivatized by methoxylation and trimethylsilylation reactions, at *m/z* 319, 307, 173 and 160. See Table I for peak identification. The various segments divided by vertical bars include one or more mixtures where the tracing of each mixture is identified by letters, by letters followed by points or by numbers.

(two peaks) and rhamnose (one peak). Except for ribose, the resolution of peaks for individual pentoses and pentitols in mixtures was unsatisfactory under the conditions used for the chromatograms in Fig. 1. A less rapid increase in temperature differentiated (Table II) the minor peaks of xylose, lyxose and arabinose, the major peaks of ribose and the single peaks of xylulose, ribulose and xylitol, but the peaks of arabitol and adonitol had coincident retentions.

TABLE II

DIFFERENTIATION OF MONOSACCHARIDES WITH LESS THAN SIX CARBONS BY RELATIVE RETENTION TO XYLOSE MAJOR PEAK WITH SP-2250 GLASS CAPILLARY COLUMN* AND SP-2100 FUSED SILICA COLUMN**

Sugar	Relative retention of peaks***			
	SP-2250		SP-2100	
	1	2	1	2
Erythrol	0.775		0.512	
Xylose	(0.977)	1.000	(0.954)	1.000
Lyxose	(0.963)	0.998	(0.965)	1.029
Arabinose	(0.990)	0.999	(1.030)	1.042
Ribose	(1.007)	1.016	1.114	1.130
Xylulose	1.060		1.095	
Ribulose	1.036		1.107	1.118
Xylitol	1.027		1.333	
Arabitol	1.043		1.428	
Adonitol	1.043		1.467	

*Temperature programme: 75 to 100°C at 2°C/min and to 150°C at 10°C/min; t_R of xylose major peak: 24.4 min.
**Isothermally at 140°C; t_R of xylose major peak: 41.2 min.
***Underlined values identify unresolved peaks; values in parentheses identify minor peaks (intensity about 20—30% of major peak).

As shown in the fourth segment of Fig. 1, gluconic acid eluted after hexoses and hexitols and was followed by several peaks from glucuronic acid; sedoheptulose eluted after the first peak of glucuronic acid according to measurements (not shown) at *m/z* 243 and 317.

Finally, as shown in the last segment of Fig. 1, inositol was followed by mannoheptulose (one peak), glucoheptulose (two peaks), N-acetylglucosamine (two peaks), N-acetylmannosamine (two peaks) and N-acetylgalactosamine (shouldered peak).

Fused-silica column gas chromatography

The major peak of glucose (central portion of Fig. 2) was completely separated from all other sugars while its minor peak only lacked baseline resolution adjacent to the minor peak of galactose. The major peaks of galactose and mannose were well resolved. Tagatose and fructose showed no intensity at *m/z* 319 and 160, but each produced two major peaks that were well resolved; at *m/z* 307 and 173, a third very minor peak arising from the tagatose sample might be a sorbose contaminant. Sorbose from a separate mixture

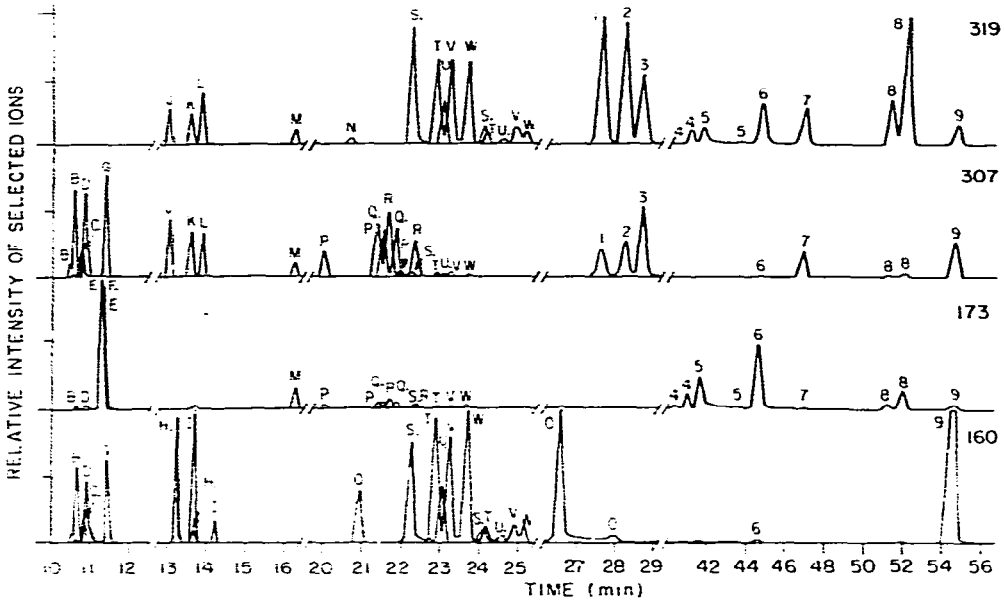


Fig. 2. Fused silica (SP-2100) gas chromatography of various sugars derivatized by methoxylation and trimethylsilylation reactions, at *m/z* 319, 307, 173 and 160. See Table I for peak identification. The various segments divided by vertical bars include one or more mixtures where the tracing of each mixture is identified by letters, by letters followed by points or by numbers.

containing a few other monosaccharides showed two peaks that would fuse on one side with a tagatose peak and on the other with a fructose peak. The major peak of allose was completely separated from all other peaks at *m/z* 319 and 160 but gave a little residual intensity coinciding with the second peak of fructose. Talose, which is unlikely to be found naturally in biological samples, would interfere with measurements of areas but not peak heights of the major peaks of mannose and galactose. Sedoheptulose gave no intensity in this chromatogram, but at *m/z* 333 (not shown) produced a peak coinciding with the second peak of allose. At *m/z* 160 one major peak from glucuronic acid was perfectly resolved from all others, and a minor peak eluted at the same time as the major peak of glucose. At *m/z* 319, a first peak from glucuronic acid preceded all the hexoses, a second peak without intensity at *m/z* 319, but monitored at *m/z* 244 (not shown) eluted at the same time as the first peak of tagatose; two other peaks from glucuronic acid were detected following hexoses but only at *m/z* 160.

In contrast to chromatography with the glass capillary column, the hexitol peaks seen at *m/z* 319 and 307 eluted well after the hexoses. On the other hand, hexoses were preceded by 2-deoxy-D-glucose (single peaks at *m/z* 319, 307 and 173) and the 6-deoxysugars which gave peaks only at *m/z* 160 where the major peak of fucose coincided with the minor peak of rhamnose. As with the glass capillary column, and except for ribose, unsatisfactory separations of individual peaks of pentoses and pentitols were obtained with the fused silica column at 180°C, but all could be differentiated at 140°C (Table II) although the major peak of lyxose coincided with the minor peak of arabinose.

The last segment of Fig. 2 shows well separated peaks: two from both N-acetylglucosamine and N-acetylmannosamine, one from both N-acetylgalactosamine and inositol, two from mannoheptulose and one from glucoheptulose.

In a different type of application where differentiation of monosaccharides and the corresponding alditols was not required, and the former were reduced to the latter, Bradbury et al. [13] reported a comparable resolution of TMS alditols with a Perkin-Elmer OV-101 fused silica column. This resolution had a considerably longer t_R than in the present study with a 50-m column, even at a temperature 10°C higher.

In general, compared with the SP-2250 glass capillary column, the SP-2100 fused silica column not only gave a better overall resolution of the various sugars but also had the advantage of being easy to install without the risk of breakage associated with glass capillary columns.

Quantitative analysis with SP-2100 fused silica capillary column

Analysis of the deuterium-labelled algal sugar mixture used for isotope ratios calibration identified the presence of various deuterated sugars (Fig. 3).

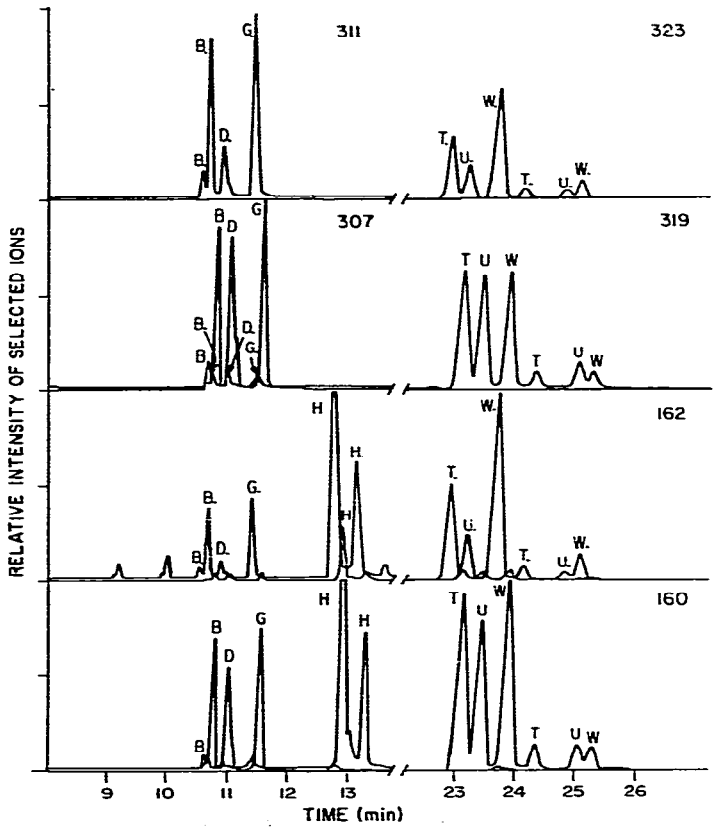


Fig. 3. Fused silica (SP-2100) gas chromatography of trimethylsilyl-O-methyloxime derivatives of a deuterated algal sugar mixture (letters followed by full points) and of a mixture of non-deuterated sugars corresponding to identified deuterated sugars. See Table I for peak identification. The tracings for each mixture are superimposed.

TABLE III

RECOVERIES OF GLUCOSE, MANNOSE, GALACTOSE, FRUCTOSE AND XYLOSE ADDED TO SERUM DEPLETED OF GLUCOSE* AS DETERMINED BY ISOTOPE RATIOS** FOLLOWING CHROMATOGRAPHY WITH FUSED SILICA COLUMN

Added (mg/dl)	Recovery (%)				
	Glucose	Galactose	Mannose	Fructose	Xylose
200	100.2				
100	101.4	102.7	100.7	99.6	
50	100.4				
10		100.9	109.4	101.0	100.7
2		100.2	102.1	99.7	98.0

*Yeast-treated serum [14] containing 1.2 mg/dl of glucose and 0.3 mg/dl of mannose.
**Peak-area ratios of major peaks of glucose, galactose, mannose at *m/z* 319 and of fructose at *m/z* 307 over area at *m/z* 323 for deuterated glucose and the ratio of the xylose peaks at *m/z* 275 over those for deuterated xylose at *m/z* 311.

On the basis of peak-area measurements compared with known amounts of the corresponding unlabelled sugars, these gave the following concentrations in g/dl of the original ampoule: xylose 0.7, arabinose 0.2, ribose 0.7, rhamnose 1.4, mannose 3.4, galactose 2.0 and glucose 6.0. As some sera may contain enzymes capable of isomerizing some of these sugars at room temperature, precautions must be taken to avoid this possible source of error by maintaining the vials to which serum has been added at 5°C during the addition of the labelled sugar mixture and freezing immediately after.

A limited study was made concerning the applicability of the above technique to the determination of glucose, galactose, mannose, fructose and xylose in serum by isotope dilution analysis following chromatography of MO-TMS derivatives with a fused silica column. Analysis of a yeast-treated serum [14] showed only traces of glucose (1.2 mg/dl) and mannose (0.3 mg/dl). Table III shows complete recoveries of various concentrations of glucose, galactose, mannose, fructose and xylose added to that serum depleted of sugar. While much more work and data are still required concerning accuracy and precision in order to be able to recommend specific techniques for sample preparation and SIM measurements, the above results are indicative of the potential usefulness of the combination of a MO-TMS derivatization technique [4], SP-2100 fused silica column chromatography and SIM for the determination of numerous monosaccharides and related sugars.

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