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APPLICATION OF GAS-LIQUID CHROMATOGRAPHY TO THE STRUCTURAL INVESTIGATION OF POLYSACCHARIDES—I

THE STRUCTURES OF THE GUMS OF *VIRGILIA OROBOIDES* AND OF *AGAVE AMERICANA*

A. M. STEPHEN, M. KAPLAN,† G. L. TAYLOR and E. C. LEISEGANG
Department of Chemistry, University of Cape Town, S. Africa

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Abstract—The use of gas-liquid chromatography for the separation of the methyl ethers of sugars as their methyl glycosides provides a rapid and convenient method for the analysis of the cleavage products from methylated polysaccharides. The relative molar responses of a series of methyl glycosides have been determined using a flame ionization detector, and the results suggest that the magnitude of the response is related to the retention time. The analyses of *Virgilia oroboides* gum and *Agave americana* gum are reported and compared to previously published results. It is concluded that in this type of investigation the error in the chromatographic analysis itself is no greater than that which accrues from the methylation and methanolysis procedures during the preliminary working-up.

INTRODUCTION

BISHOP[1] in 1958 first described the separation of fully methylated methyl glycopyranosides by GLC. Rapid progress was made in extending the technique to the separation of other carbohydrate derivatives[2,3] by several authors[4–6] and the subject has recently been reviewed.[7]

Quantitative analyses of such glycosides have been carried out using the β-ray ionization detector and it has been shown that some fully acetylated glycitols give rise to a response the magnitude of which is linearly related to the number of moles of glycoside present[8] but that di-O-methyl hexosides give rise to a lower response than the corresponding tri- and tetra-O-methyl hexosides.

In this paper the qualitative and quantitative analysis of sugar glycosides, using a flame-ionization detector, is reported, and the method is illustrated by the analysis of methanolysis products from *Agave americana* and *Virgilia oroboides* gums.

* This paper is dedicated to the memory of Professor H. Stephen.

† Present address: Organic Chemical Laboratories, University of Cambridge, England.

[1] C. T. Bishop, F. P. Cooper, D. H. Ball and A. G. McInnes, *J. Chromatog.* **1**, 556 (1958).

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[5] R. Kuhn and H. Egge, *Chem. Ber.* **96**, 3338 (1963).

[6] T. Yamakawa and N. Ueta, *Jap. J. Exp. Med.* **34**, 37 (1964).

[7] C. T. Bishop, *Adv. Carbohydrate Chem.* **19**, 95 (1964).

[8] W. J. A. van den Heuvel and E. C. Horning, *Biochem. Biophys. Res. Comm.* **4**, 399 (1961).

PROCEDURE

The instrument used was a Beckmann GC-2A gas chromatograph fitted with flame-ionization detector. A three foot column, loaded with ethylene glycol succinate polyester on Chromosorb W (80–100 mesh), was used at 155°. The carrier gas was He and the inlet press. 23 p.s.i.

The loading of the column was initially 14%, but there is evidence that some of the polyester was bled off under operating conditions.[9] The conditions used resemble those used by Aspinall[10] (butan-1,4-diol succinate polyester at 175°).

TABLE 1. RELATIVE RETENTIONS (*T*) OF METHYL GLYCOSIDES

Methylated sugar	*T* values			
2,3,4,6-Tetra-O-methylglucose	1·00 m	1·52 s		
2,3,4,6-Tetra-O-methylgalactose	2·00			
2,3,4-Tri-O-methylgalactose	9·3 sh	9·8		
2,3,6-Tri-O-methylgalactose	3·90 s	4·77 w	5·48 w	6·20 m
2,4,6-Tri-O-methylgalactose	5·12 m	6·08 s		
2,3-Di-O-methylgalactose	14·6 s	18·3 w	19·7 w	25·3 w
2,6-Di-O-methylgalactose	14·1 s	17·3 w	20·1 m	26·9 vw
2,4-Di-O-methylgalactose	25·5 m	30·0 s		
2,3,4,6-Tetra-O-methylmannose	1·54			
3,4,6-Tri-O-methylmannose	3·74			
4,6-Di-O-methylmannose	16·4			
2,3,4-Tri-O-methylrhamnose	0·44			
3,4-Di-O-methylrhamnose	1·10			
3-O-methylrhamnose	4·86			
2,3,4-Tri-O-methylarabinose	1·26			
2,3,5-Tri-O-methylarabinose	0·59 s	0·79 w		
2,3-Di-O-methylarabinose	1·96 s	2·29 w	2·54 m	
2,4-Di-O-methylarabinose	2·94 sh	3·16		
2,5-Di-O-methylarabinose	2·33 s	4·63 w		
3,4-Di-O-methylarabinose	2·82			
3,5-Di-O-methylarabinose	1·28	3·21		
3-O-Methylarabinose	4·55 w	6·57 s	11·1 w	14·6 m
2,3,4-Tri-O-methylxylose	0·44 m	0·59 s		
2,3-Di-O-methylxylose	1·75 m	1·95 w	2·20 s	
2-O-Methylxylose	5·88	9·35		
3-O-Methylxylose	5·00 s	8·12 m		
2,3,4-Tri-O-methylglucuronic acid	2·78 m	3·72 s		
2,3-Di-O-methylglucuronic acid	11·7 s	15·8 m		

Note: (1) The retention time of 2,3,4,6-tetra-O-methylglucose (*T*, 1·00) was 4 mins.
(2) The uronic acids were present as methyl esters.
(3) Symbols used: s = strong; m = medium; w = weak; vw = very weak; sh = shoulder.

Standard sugars were converted to their methyl glycosides by heating with methanolic HCl (100°, 6 hr) and the retention times of the glycosides, relative to methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside (*T*, 1·00) as internal standard are given in Table 1. These *T* values were found to be reproducible to within 2%, and when plotted logarithmically against Aspinall's *T* values, linear relationships were observed. This not only provided additional support for the authenticity of the standard sugars, but also made possible the identification of peaks in subsequent analyses wherever standard sugars were not obtainable.

The response of the flame-ionization detector has been shown to be linearly related to sample size for a wide range of compounds. This linearity has been confirmed for methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside and has been assumed for the remainder of the glycosides used.

[9] M. Kaplan, M. Sc. Thesis, University of Cape Town (1965).
[10] G. O. Aspinall, *J. Chem. Soc.* 1676 (1963).

EXPERIMENTAL

Methylation and methanolysis procedures for gum. Gum in the form of colourless translucent nodules, collected from wound marks on the fleshy leaves of *Agave americana* (Agavales-Agavaceae) was purified in the usual way by EtOH precipitation from aqueous solution; it had $[\alpha]_D$ −30° (*c* 1·0 in water) and equiv. (by titration), 1050. Acid hydrolysis indicated galactose and arabinose, less of rhamnose, and the aldobiouronic acid (6-D-galactose-β-D-glucopyranosid)-uronic acid. Methylation (successive procedures of Haworth, Kuhn and Purdie) until no increase in the methoxyl (36·9%) could be effected gave, after dialysis, an ash-free product in good yield, $[\alpha]_D$ −63° (*c* 4·7 in $CHCl_3$).

TABLE 2. RELATIVE MOLAR RESPONSE OF GLYCOSIDES

Glycoside	*R* experimental	*R* modified
2,3,4,6-Tetra-O-methylglucose	1·00	1·00
2,3,4,6-Tetra-O-methylgalactose	0·93	0·93
2,3,4-Tri-O-methylgalactose	0·69	0·58
2,4,6-Tri-O-methylgalactose	0·62	0·69
2,3,6-Tri-O-methylgalactose	0·77	0·73
2,6-Di-O-methylgalactose	0·50	0·55
2,4-Di-O-methylgalactose	0·41	0·41
2,3,5-Tri-O-methylarabinose	1·07	1·08
2,3-Di-O-methylarabinose	0·51	0·68
2,5-Di-O-methylarabinose	0·64	0·62
3,5-Di-O-methylarabinose	0·63	0·62
3-O-Methylarabinose	0·23	0·22
2,4-Di-O-methylarabinose	0·56	0·56
3,4-Di-O-methylarabinose	0·61	0·60
3,4,6-Tri-O-methylmannose	0·65	0·65
2,3,4-Tri-O-methylglucuronic acid	0·54	0·54
2,3-Di-O-methylgalactose	—	0·44
2,3,4,6-Tetra-O-methylmannose	—	0·89
4,6-Di-O-methylmannose	—	0·25
2,3,4-Tri-O-methylarabinose	—	0·86
2,3,4-Tri-O-methylrhamnose	—	1·20*
3-O-Methylrhamnose	—	0·43*
2,3,4-Tri-O-methylxylose	—	1·06
2,3-Di-O-methylglucuronic acid	—	0·38*

* Calculated by assumption that the relationship between *R* and log *T* is valid.

(Found: C, 52·6; H, 7·9%.) The IR spectrum confirmed the extent of methylation (freedom from OH absorption), as well as the order of magnitude of the equiv. wt. (from the absorption of the ester carbonyl). Paper chromatography of the acid hydrolysate of the methylated gum indicated a range of sugars compatible with those found subsequently in the methanolysate. Methanolysis of methylated *Agave americana* gum (2·4% methanolic HCl, sealed tube, 18 hr at 100°), neutralization (Ag_2CO_3), and GLC of the product led, by use of corrected *R* values, to the result shown in Table 3. (Methyl esters were removed by saponification of the methanolysate in order to assist in the analysis.)

Quantitative analysis. Relative molar responses (*R* values) of the sugar glycosides were determined by heating an accurately weighed quantity of sugar (1–10 mg) together with a similar quantity of 2,3,4,6-tetra-O-methyl-D-glucose in methanolic HCl for 6 hr. After being neutralized (Ag_2CO_3) the methanolic solution was subjected to GLC, and peak areas were determined by triangulation.

The *R* value was defined as follows:

$$R = \frac{\text{sum of peak areas of methyl glycosides per mole of sugar}}{\text{sum of peak areas of methyl glycosides per mole of 2,3,4,6-tetra-O-methyl-D-glucose}}$$

The experimental *R* values are presented in Table 2. 2,3,4,-Tri-O-methyl-D-glucuronic acid was not

available as a standard, but on GLC of the methanolysates from (6-D-galactose-β-D-glucopyranosid)-uronic acid and (2-D-mannose-β-D-glucopyranosid)-uronic acid one could deduce the extent of cleavage of the uronic acid–sugar bonds (from appropriate known R values) and hence one could obtain the R value of the common uronic acid.

An apparent relationship was observed to exist between the experimental R and T values. By plotting R vs. log T, the experimental data fell on two straight lines, one for glucose and galactose

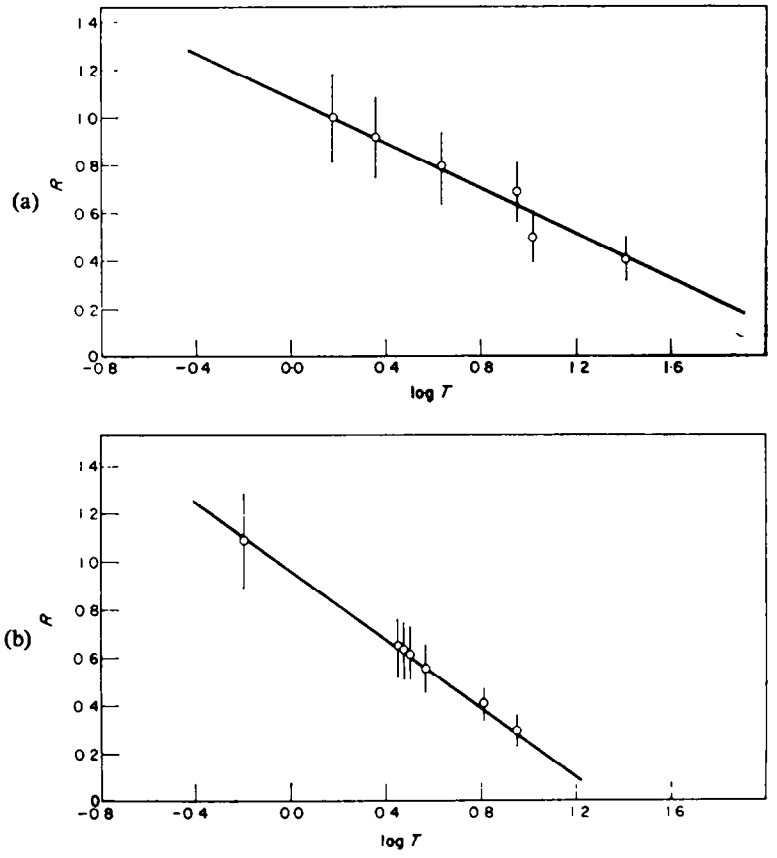

FIG. 1. Apparent relationship between molar response and relative retention time.
1a Methyl ethers of glucose and galactose.
1b Methyl ethers of arabinose and xylose.

derivatives, the other for arabinose and xylose. This is illustrated in Fig. 1. Mannose was found to have an anomalous position. In many cases a given glycoside gave rise to more than one peak—a well-known phenomenon attributed to different anomeric forms. In such cases, the value of T used was an average value, given by:

$$T' = \Sigma a_i T_i / \Sigma a_i$$

a_i = area of peak i
T_i = relative retention time of peak i.

The shaded areas around the points in Fig. 1 represent the 95% confidence limits of the quantitative analysis. It is clear that the T values are very highly reproducible but that the R values are a good deal less accurately known. The relatively large uncertainty arises from (a) the difficulty of preparing pure standard methyl glycosides, (b) demethylation and degradation of the methylated sugars occurring during methanolysis, (c) the errors involved in measuring the areas of incompletely resolved peaks.

If one assumes that the linear relationships in Fig. 1 are correct, and that the slope of the line is known much more accurately than the positions of the points, then the experimental *R* values may be modified to fit the observed line. The modified *R* values thus obtained as well as the experimentally determined values are shown in Table 2.

TABLE 3. ANALYSIS OF *Virgilia oroboides* AND *Agave americana* GUMS

Sugar	Legend	*Virgilia oroboides*		*Agave americana*
		Mole percentage by GLC	Mole percentage (Literature)[11]	Mole percentage by GLC
2,3,4-Tri-O-methylxylose	a	1	1	
2,3,5-Tri-O-methylarabinose	c	7	12	12
2,3,4-Tri-O-methylarabinose	d	6	7	
2,3-Di-O-methylarabinose	e	8	18	
2,3,4,6-Tetra-O-methylgalactose	j	2	2	6
2,4,6-Tri-O-methylgalactose	l	4	2	4
2,3,4-Tri-O-methylgalactose	m	41	24	9
2,3-Di-O-methylgalactose	n	5	7	
2,4-Di-O-methylgalactose	p	15	12	27
4,6-Di-O-methylmannose	r	3	4	
2,3,4-Tri-O-methylglucuronic acid	u	3	6	9
2,3-Di-O-methylglucuronic acid	v	6	3	10
2,3,4-Tri-O-methylrhamnose	b			10
3,5-Di-O-methylarabinose	f			2
2,5-Di-O-methylarabinose	g			2
2,6-Di-O-methylgalactose	o			1
Unidentified	x			3

RESULTS

The *R* values obtained were used in quantitative analyses of methanolysis products from a number of methylated polysaccharides, two of which are reported here. The molar proportions of sugar residues in the methanolysates from methylated *Agave americana* and *Virgilia oroboides* gums were obtained by dividing the peak areas of the sugar methyl glycosides by their *R* values. The chromatograms are reproduced in Figs. 2 and 3: the resulting analyses are presented in Table 3 where they are compared with previously published values.

Virgilia oroboides. It is interesting to compare these results with the structural model proposed for the polysaccharide gum exudate from the bark of *Virgilia oroboides*, a material of interest in relation to those obtained from other representatives of the Leguminosae. This polysaccharide contains 1 → 6-linked D-galactopyranose to which groups of sugars (R)* are attached irregularly mainly at positions 3 and 4. The ratios of unbranched, →3-linked, and →4-linked galactose units were given[11] as approximately 3:2:1. Periodate oxidation and Smith degradation of the polysaccharide showed[12] the proportion of branched galactose to be a little higher than this. The

* *R* = D-gluA 1 → 2-D-Man$_p$ 1 →
D-gluA 1 → 6-D-Gal$_p$ 1 →
L-Ara$_p$ 1 → 5-L-Ara 1 →
L-Ara$_f$ 1 → 5-L-Ara 1 →

[11] A. M. Stephen, *J. Chem. Soc.* 1974 (1963).
[12] J. McD. Blair, A. M. Stephen and (in part) D. H. Shaw, *J. S. Afr. Chemical Inst.* **18**, 28 (1965).

analytical results now obtained, using GLC separation and assay, are consistent with the published findings but show a higher proportion of 2,3,4-tri-O-methylgalactose is present, and less of 2,3,5-tri-O-methyl- and 2,3-di-O-methylarabinose. Cellulose column chromatography had proved ineffective in separating 2,3,4-tri-O-methyl-galactose and 2,3,-di-O-methylarabinose in the earlier work, and it was partly for this reason that the GLC method of analysis was undertaken. The totals for these components are similar as determined by the two methods; the effects of changing the proportions of →6-linked galactose and →5-linked arabinose upon the model structure

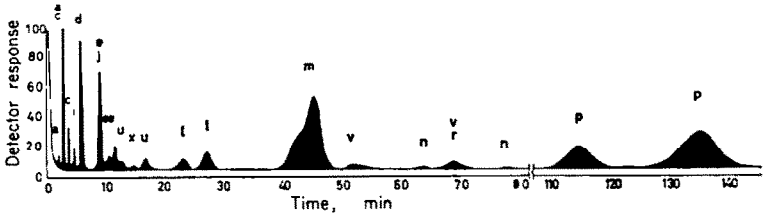

FIG. 2. Chromatogram of methanolysate from *Virgilia oroboides* gum.

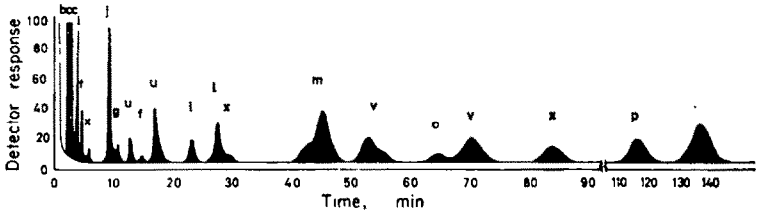

FIG. 3. Chromatogram of methanolysate from *Agave americana* gum.

is to increase the contribution of unbranched galactose and to decrease correspondingly the unbranched arabinose chain units. Both yield glycerol upon periodate oxidation, etc., of the gum.

A low proportion of 2,3,5-tri-O-methylarabinose has been accounted for[13] by assuming that acid-labile arabinofuranose residues are lost whilst methylating the gum; the same reason applies here.

Agave americana. In addition to the interest in the sapogenin present in sisal leaves, studies have also been carried out on the lignin in the holocellulose,[14] and on the pectic components[15] and hemicelluloses.[16] Furthermore, a preliminary study of a sisal gum has very recently been reported.[17]

The structure of *Agave americana* gum bears a close resemblance to those of the *Acacias*. The constituent sugars are the same (D-galactose, D-glucuronic acid, L-arabinose and L-rhamnose*), the acid being bound $\beta 1 \rightarrow 6$ to galactose as is usual.

* Note that the enantiomeric forms are assumed in this report to be those found commonly in materials of this type.

[13] A. M. Stephen, *J. Chem. Soc.* 2030 (1962).
[14] M. Luedtke, *Holzforschung* **15**, 161–8 (1961).
[15] G. O. Aspinall and C. Rodriguez, *J. Chem. Soc.* 4020 (1958).
[16] P. P. Mukherjee, *Textile Res. J.* **34**, (2) 179 (1964).
[17] P. Hope, H. S. de Leon and A. Benitez, *Anales Escuela Nacl. Cienc. Biol.* (Mex) **11** (1–4), 3–13 (1962). *Chem. Abstr.* **61**, 8621 h.

The resemblance to the gum of *Acacia cyanophylla*[18] at present being studied in some detail in this laboratory[19] is particularly marked, not only in respect of the proportions of the component sugars and the specific rotations of the intact polysaccharide molecules, but also in the manner in which the individual sugar units are linked.

Like *Acacia cyanophylla*, *Agave americana* contains the galactose (pyranose form) predominantly as branch-points (linked through $C_{(3)}$ and $C_{(6)}$) also as non-reducing end groups, as chain units linked through $C_{(6)}$ and $C_{(3)}$, and to a small extent as double-branch points. The high proportion of uronic acid occurs partly as end groups, the rest linked through $C_{(4)}$. The sugar responsible for this in *Acacia cyanophylla* is L-rhamnose; in *Agave americana* the 1:1 molar ratio of rhamnopyranose to 4-linked glucuronic acid suggests a similar relationship between the methylpentose and the acid components of the gum. The total rhamnose content of *Agave americana* gum is markedly less than that of *Acacia cyanophylla*. The remainder of the material consists of arabinose in a fairly low proportion overall, most of it as furanose end-groups, trans linked through $C_{(3)}$ and $C_{(2)}$.

The finding of so many points of similarity between gums from plants in different orders is in conformity with earlier observations that although similarities may be expected in gums from different species within a genus, they may also be found in widely scattered parts of the plant kingdom.[20]

A further application of GLC to the quantitative analysis of the *Acacia* gums is discussed in an accompanying paper.

DISCUSSION

It is clear that GLC is no substitute for classical methods of structure determination, but although no detailed proof of the structures of these two gums has been presented, a great amount of structural information has been collected very rapidly using small quantities of starting material.

Regarding the retention behaviour, it is interesting to note that the theory of Calcote[21] which explains the response of compounds to the flame ionization detector by a chemi-ionization mechanism, is apparently not applicable to sugar glycosides. Although it correctly predicts that the response should increase with increasing methylation, it also predicts that the response of isomeric sugar glycosides should be identical. This is not supported by the present work.

The conditions used in this study have the practical advantage that retentions are not excessively long even for di-O-methylhexosides, while at the same time selectivity is good even for the fully methylated glycosides. Earlier workers have generally needed to use two columns of differing polarity to realize both of these advantages.

The apparent relationships between R and $\log T$ are interesting, because if these can be shown to be general, they will be of value in the quantitative analysis of all polysaccharides. Although the experimental data seem to indicate that the relationships are real, and although an explanation can be rationalized,[9] it should be pointed out that no attempt has been made to collect and characterize the glycosides eluted from

[18] A. J. Charlson, J. R. Nunn and A. M. Stephen, *J. Chem. Soc.* 269 (1955).
[19] A. J. Charlson, M. Kaplan and A. M. Stephen, unpublished results.
[20] A. M. Stephen and E. A. C. L. E. Schelpe, *S. Afr. Ind. Chemist* 12 (1965).
[21] H. F. Calcote, *Combustion and Flame* **1**, 385 (1957).
[22] A. M. Stephen, *S. African Ind. Chemist* 83 (1963).

the column, furthermore the possibility of ester interchange or some other mode of decomposition of the glycosides on the column cannot be discounted.

Three samples of the methylated disaccharide 3-O-α-D-galactopyranosyl-L-arabinose were heated individually with methanolic hydrogen chloride in sealed tubes. The methanolysates were analysed by GLC and the total peak areas of the methyl glycosides of 2,4-di-O-methylarabinose and 2,5-di-O-methylarabinose determined from the chromatograms. The 95% confidence limits for the relative peak areas of these sugars were found to be ±24% and ±21% respectively, whereas the 95% confidence limit for the *reproducibility* of the peak areas in any one methanolysate was found to be ±2%.

From this one must conclude that the errors involved in methanolysis procedure (for example demethylation) are far more serious than the errors encountered in the gas-chromatographic analysis. This would be particularly true in the case of a polysaccharide in which methylation might be incomplete, degradation might occur during methylation and cleavage of uronic acid-sugar bonds during methanolysis might also be incomplete.

For this reason it is felt that it would be futile to refine the analytical technique without first eliminating the major sources of error in the working-up procedure.

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