## 314. *Gas–Liquid Partition Chromatography of Methylated and Partially Methylated Methyl Glycosides.*

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Gas–liquid partition chromatography of methylated and partially methylated methyl glycosides provides a highly selective method for the analysis of individual methylated sugars and of the cleavage products from methylated oligo- and poly-saccharides. The relative retention times of a large number of such methyl glycosides on two stationary phases are reported. The value of this technique for the preliminary identification of complex mixtures of methyl glycosides is illustrated by examination of the methanolysis products from methylated polysaccharides of known structure.

The first reported use of gas–liquid partition chromatography for the separation of carbohydrate derivatives was by Bishop and his collaborators [1] for fully methylated methyl glycosides. The technique has been subsequently extended to the separation of fully and partially methylated methyl glycosides by Bishop and Cooper,[2] Kircher,[3] Klein and Barter,[4] and Gee and Walker,[5] and has been employed by Adams and Bishop and their collaborators [6] in several structural studies on polysaccharides. Bishop [7] has recently reviewed the use of gas chromatography for the separation of these and other carbohydrate derivatives. Here we discuss the scope of the technique and report the relative retention times of a wide range of fully and partially methylated methyl glycosides.

Analyses were performed on a " Pye Argon Chromatograph " fitted with an ionisation detector, and the separations were carried out on samples ($2—10 \times 10^{-6}$ g.) which were injected in chloroform or methanol solution. The operating conditions were essentially those described by Bishop and Cooper,[2] and two stationary phases were used: (*a*) butan-1,4-diol succinate polyester [2] at 175°, and (*b*) polyphenyl ether [*m*-bis-(*m*-phenoxyphenoxy)-benzene] at 200°. The former and more polar liquid phase is highly selective [2] for the separation of methyl glycosides which are fully etherified or contain only one hydroxyl group, and we have found that this also has value for the methyl glycosides of mono-*O*-methylpentoses and 6-deoxyhexoses. The methyl glycosides of di-*O*-methylhexoses, however, were found to be strongly retained, and their retention times were too great to be of value for diagnostic purposes. The second and less polar liquid phase, although generally less selective, could be used for a similar range of derivatives of pentoses and 6-deoxyhexoses, and for the methyl glycosides of tetra-, tri-, and di-*O*-methylhexoses.

In a few cases anomerically pure methyl glycosides of methylated or partially methylated sugars were available as reference compounds, but in general equilibrium mixtures of methyl glycosides were formed by heating the sugar with methanolic hydrogen chloride. This procedure resulted in the formation of two, and in certain cases four, methyl glycosides, and we found that the proportions of the various methyl glycosides (as judged by peak heights or areas) as well as the relative retention times of the individual components were characteristic of the parent sugar and provided a useful " fingerprint " for diagnostic purposes. The retention times of the various methyl glycosides are most conveniently expressed in relation to an internal standard, and the relative retention times ($T$) indicated in the following Tables are based on methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside ($T = 1{\cdot}00$) as the standard. In our experience the $T$ values are

[1] McInnes, Ball, Cooper, and Bishop, *J. Chromatography*, 1958, **1**, 556.
[2] Bishop and Cooper, *Canad. J. Chem.*, 1960, **38**, 388.
[3] Kircher, *Analyt. Chem.*, 1960, **32**, 1103.
[4] Klein and Barter, *Textile Research J.*, 1961, **31**, 486.
[5] Gee and Walker, *Analyt. Chem.*, 1962, **34**, 650.
[6] Adams and Bishop, *Canad. J. Chem.*, 1960, **38**, 2380; Bishop, Blank, and Gardner, *ibid.*, p. 869; Bishop and Cooper, *ibid.*, p. 793.
[7] Bishop, *Methods of Biochemical Analysis*, 1962, **10**, 1.

reproducible to within 2% or better on individual columns, although slightly greater variations (but not more than 5%) may be encountered on different columns containing the same stationary phase. In general, components whose retention times differ by 5% or more can be resolved sufficiently to indicate the presence of both components.

Table 1 lists the relative retention times ($T$) of the various methyl glycosides obtained when the sugars at our disposal were heated with methanolic hydrogen chloride. Gas chromatography of the products from certain sugars showed only one peak, presumably due either to the anomeric glycosides having the same retention times on the particular stationary phase at the chosen operating temperature or to the formation of one substantially pure anomer. In the case of methyl glycosides of D-mannose Bishop [7] has shown that the α-glycoside is formed almost exclusively. The equilibrium mixtures of methyl glycosides of those methyl ethers of L-arabinose, D-galactose, and D-fructose, *e.g.*, 2,3-di-*O*-methyl-L-arabinose, 2,3,6-tri-*O*-methyl-D-galactose, and 1,3,4-tri-*O*-methyl-D-fructose, which are capable of forming both furanosides and pyranosides, were shown to contain more than two components, indicating the greater relative stabilities of the methyl furanosides of these sugars than those of derivatives of D-glucose, which gave rise to only two components, presumably the methyl pyranosides. In the case of 1,3,4-tri-*O*-methyl-D-fructose five components were observed on column *b* when the sugar was treated with methanolic hydrogen chloride, and it would seem possible that some open-chain dimethyl ketal was formed in addition to the four methyl glycosides. In general it was noted that methyl ethers of D-galactose and D-fructose which could form methyl furanosides, wholly or in part, gave components of markedly lower retention times than the isomeric methyl ethers which formed pyranosides only. This phenomenon was most marked on column *b* where, for example, the components of lowest retention times in the equilibrium mixtures of methyl glycosides from 2,3,6-tri- and 2,3-di-*O*-methyl-D-galactose had retention times similar to those of methyl tetra- and tri-*O*-methyl-D-galactopyranosides. Although it has not yet been possible to assign the retention times of the individual components of mixtures to specific methyl glycosides these results indicate that, in general, methyl furanosides have lower retention times than those of isomeric methyl pyranosides. This generalisation may be of value in the preliminary identification of unknown methyl ethers. It is noteworthy that Gee and Walker [5] have observed that methyl tetra-*O*-methyl-D-fructofuranosides have lower retention times than those of methyl tetra-*O*-methyl-D-fructopyranosides.

It is clear from these results and from those of other workers [7] that gas–liquid partition chromatography provides a highly selective analytical method for the resolution of methylated and partially methylated methyl glycosides. Individual methylated sugars furnish equilibrium mixtures of methyl glycosides, the retention times and relative proportions of whose components suggest the probable identity and also provide a sensitive measure of the purity of the parent sugar. All the sugars, whose methyl glycosides were examined, have been characterised as crystalline derivatives. In several cases where the sugars themselves were not crystalline, gas chromatography, by virtue of its greater selectivity, indicated the presence of appreciable proportions of impurities, although the sugars were apparently homogeneous on paper chromatography. In cases where both columns could be satisfactorily used, the methyl glycosides derived from mixtures of two or three methylated sugars were adequately resolved to permit identification of the parent sugars. It is possible, therefore, to obtain considerable information on the structures of di- and tri-saccharides by methylating milligram quantities and examining the methanolysis products from the methylated oligosaccharides by gas chromatography. Although such results alone should be considered with caution, yet when supported by independent evidence, *e.g.*, paper chromatography and ionophoresis of the hydrolysis products of the oligosaccharides and the derived glycitols, together with analysis by periodate [8] and lead

[8] Bobbitt, *Adv. Carbohydrate Chem.*, 1956, **11**, 1; Bouveng and Lindberg, *ibid.*, 1960, **15**, 75.

TABLE 1.

Relative retention times ($T$) of methyl glycosides.

| Sugar | Column *a* | | Column *b* | |
|---|---|---|---|---|
| 2,3,4-Tri-*O*-methyl-L-arabinose | 1·04 | — | 0·83 | — |
| 2,3,5-Tri-*O*-methyl-L-arabinose | 0·56s | 0·72m | 0·47s | 0·59m |
| 2,3-Di-*O*-methyl-L-arabinose | 1·56s | 1·76w | 0·63s | 0·82w |
| | 1·93m | — | 0·95m | — |
| 2,4-Di-*O*-methyl-L-arabinose | 2·26sh | 2·37 | 1·09sh | 1·13 |
| 2,5-Di-*O*-methyl-L-arabinose | 1·89s | 3·47w | 0·70s | 1·03w |
| 3,4-Di-*O*-methyl-L-arabinose | 2·15 | — | 0·99 | 1·52 |
| 3,5-Di-*O*-methyl-L-arabinose | 1·08 | 2·55 | 0·60 | 0·84 |
| 2-*O*-Methyl-L-arabinose | 6·1 | — | 1·10 | 1·45 |
| 3-*O*-Methyl-L-arabinose | 3·42w | 4·47s | 0·88w | 1·26s |
| | 6·95m | — | 1·45m | 1·58m |
| 2,3,4-Tri-*O*-methyl-D-xylose | 0·46m | 0·57s | 0·45m | 0·54s |
| 2,3-Di-*O*-methyl-D-xylose | 1·50m | 1·65w | | |
| | 1·79s | — | 0·64m | 0·75s |
| 2,4-Di-*O*-methyl-D-xylose | 1·49m | 1·97s | 0·73m | 0·93s |
| 3,4-Di-*O*-methyl-D-xylose | 1·36s | 1·63m | 0·71s | 0·76m |
| 2-*O*-Methyl-D-xylose | 4·11 | 6·23 | 1·01 | 1·34 |
| 3-*O*-Methyl-D-xylose | 3·55s | 5·57m | 0·94s | 1·15m |
| 4-*O*-Methyl-D-xylose | 4·35 | — | 1·09 | — |
| 2,3,4-Tri-*O*-methyl-L-rhamnose | 0·46 | — | 0·46 | — |
| 3,4-Di-*O*-methyl-L-rhamnose | 0·73w | 1·01s | 0·61 | — |
| 3-*O*-Methyl-L-rhamnose | 3·66 | — | 1·01 | — |
| 2,3,4-Tri-*O*-methyl-L-fucose | 0·72 | — | | |
| 2-*O*-Methyl-L-fucose | 4·25 | — | 0·99s | 1·15m |
| | | | 1·133s | 1·54w |
| 2,3,4,6-Tetra-*O*-methyl-D-glucose | 1·00m | 1·43s | 1·00m | 1·32s |
| 2,3,4-Tri-*O*-methyl-D-glucose | 2·59m | 3·70s | 1·35m | 1·83s |
| 2,3,6-Tri-*O*-methyl-D-glucose | 3·52m | 4·78s | 1·71m | 2·18s |
| 2,4,6-Tri-*O*-methyl-D-glucose | 3·31m | 4·88s | 1·64m | 2·24s |
| 3,4,6-Tri-*O*-methyl-D-glucose | 3·12s | 3·73m | | |
| 2,3-Di-*O*-methyl-D-glucose | — | — | 2·46m | 3·22s |
| 2,4-Di-*O*-methyl-D-glucose | — | — | 2·30m | 3·22s |
| 2,3,4,6-Tetra-*O*-methyl-D-galactose | 1·80 | — | 1·52sh | 1·60 |
| 2,3,4-Tri-*O*-methyl-D-galactose | 7·5 | — | 2·62m | 2·89s |
| 2,3,6-Tri-*O*-methyl-D-galactose | 3·21s | 3·93vw | 1·61s | 2·07vw |
| | 4·30w | 4·70m | 2·23w | 2·49m |
| 2,4,6-Tri-*O*-methyl-D-galactose | 4·17m | 4·70s | 2·08m | 2·38s |
| 2,3-Di-*O*-methyl-D-galactose | — | — | 2·46s | 3·19vw |
| | | | 3·65w | 4·20m |
| 2,4-Di-*O*-methyl-D-galactose | — | — | 3·72m | 4·40s |
| 2,6-Di-*O*-methyl-D-galactose | — | — | 2·51s | 3·21m |
| | | | 3·77s | — |
| 2,3,4,6-Tetra-*O*-methyl-D-mannose | 1·42 | — | 1·29 | — |
| 2,3,4-Tri-*O*-methyl-D-mannose | 3·11 | — | 1·65 | — |
| 2,3,6-Tri-*O*-methyl-D-mannose | 5·08 | — | 2·35 | — |
| 3,4,6-Tri-*O*-methyl-D-mannose | 3·08 | — | 1·71 | — |
| 2,3-Di-*O*-methyl-D-mannose | — | — | 3·37 | — |
| 3,4-Di-*O*-methyl-D-mannose | — | — | 2·26 | — |
| 1,3,4,6-Tetra-*O*-methyl-D-fructose | 1·04s | 1·26m | 1·01s | 1·16m |
| 1,3,4-Tri-*O*-methyl-D-fructose | 1·89w | 2·49s | 1·10w | 1·31s |
| | 3·94m | 4·43m | 1·71m | 2·05vw |
| | | | 2·39m | — |
| 3,4,6-Tri-*O*-methyl-D-fructose | 2·74s | 4·12m | 1·52m | 1·79s |
| 3,4-Di-*O*-methyl-D-fructose | — | — | 1·72s | 2·23m |
| | | | 3·02w | — |
| 2,3,4-Tri-*O*-methyl-D-glucuronic acid * | 2·53m | 3·24s | 1·77m | 2·21s |
| 2,3-Di-*O*-methyl-D-glucuronic acid * | 8·4m | 9·3s | 2·47m | 3·12s |
| 2,3,4-Tri-*O*-methyl-D-galacturonic acid * | 7·18 | — | 3·90s | 4·26m |

* Present as methyl esters.

The relative intensities of peaks are indicated by s = strong, m = medium, w = weak, and vw = very weak. Where incomplete resolution of peaks occurs, sh = shoulder.

tetra-acetate [9] oxidations, they provide strong evidence for the structures of di- and tri-saccharides. The value of gas chromatography in this connection has been illustrated by Perila and Bishop [10] in their characterisation of the oligosaccharides from the enzymic hydrolysis of Jack pine glucomannan, and further examples are given in two accompanying papers.[11,12]

We have examined the methanolysis products of two complex methylated polysaccharides, whose cleavage products were characterised by the formation of crystalline derivatives in previous studies.[13,14] Table 2 summarises the results in the case of methylated *Acacia pycnantha* gum. The relative retention times (*T* values) of the methyl glycosides are shown for two columns, the values in brackets indicating *T* values of components which were not resolved. The results clearly indicate the presence in the methanolysate of methyl glycosides of 2,3,4-tri-*O*-methylrhamnose, 2,3,5-tri- and 2,5-di-*O*-

TABLE 2.

Examination of methanolysis products from methylated *Acacia pycnantha* gum by gas chromatography.

| Sugars previously characterised | Approx. relative propn. | Relative retention times (*T*) of methyl glycosides: Column *a* | Column *b* |
|---|---|---|---|
| 2,3,4-Tri-*O*-methylrhamnose | + | 0·45 — | (0·46) — |
| 2,3,5-Tri-*O*-methylarabinose | +++ | 0·56, 0·72 | (0·46), 0·59 |
| 2,5-Di-*O*-methylarabinose | ++ | (1·89), 3·45 | 0·70, 1·07 |
| 2,3,4,6-Tetra-*O*-methylgalactose | +++ | (1·80) — | 1·51, 1·58 |
| 2,3,4-Tri-*O*-methylgalactose | ++ | 7·45 — | 2·60, 2·88 |
| 2,4,6-Tri-*O*-methylgalactose | ++ | 4·18, 4·73 | 2·07, 2·34 |
| 2,4-Di-*O*-methylgalactose | ++++ | — — | 3·70, 4·39 |
| 2,3,4-Tri-*O*-methylglucuronic acid | + | (2·54), 3·24 | (1·76), (2·21) |
| Sugar not previously fully characterised | | | |
| 2,3-Di-*O*-methylarabinose | + | 1·56, (1·76) (1·93) — | 0·64, 0·84 0·96 — |

methylarabinose, 2,3,4,6-tetra-, 2,3,4-, and 2,4,6-tri-, and 2,4-di-*O*-methylgalactose; the methyl glycosides of 2,3,4-tri-*O*-methylglucuronic acid were less certainly detected. Another cleavage product, which was not fully characterised previously,[13] namely, 2,3-di-*O*-methylarabinose, was indicated by the *T* values of its methyl glycosides.

The methanolysis products from methylated gum ghatti were examined similarly and the results are given in Table 3. The *T* values of the components indicate with reasonable certainty the presence of methyl glycosides of the following known cleavage products, 2,3,4-tri-*O*-methylrhamnose; 2,3,5-tri-, and 2,3-, 2,4-, and 2,5-di-*O*-methylarabinose; 2,3,4,6-tetra-, 2,3,4-tri-, and 2,4-di-*O*-methylgalactose; and 2,3,4-tri- and 2,3-di-*O*-methylglucuronic acid; the methyl glycosides of 3,5-di-*O*-methylarabinose were indicated with less certainty, and those of 2,4,6-tri-*O*-methylgalactose, a cleavage product which was not previously recognised,[14] were indicated on both columns.

It is probable that in the methanolysis of methylated acidic polysaccharides only partial cleavage of the acid-stable glycosiduronic acid linkages occurs and that the methyl glycosides of hexuronic acids are formed in low yield, so that such products may not always be readily recognised by gas chromatography. The presence of tri- and di-*O*-methylhexuronic acid residues in methylated polysaccharides can be recognised by the formation of methyl glycosides of the corresponding methylated hexoses which result

[9] Perlin, *Adv. Carbohydrate Chem.*, 1959, **14**, 9.
[10] Perila and Bishop, *Canad. J. Chem.*, 1961, **39**, 815.
[11] Aspinall and Ross, following paper.
[12] Aspinall, Cairncross, and Ross, *J.*, 1963, 1721.
[13] Aspinall, Hirst, and Nicolson, *J.*, 1959, 1697.
[14] Aspinall, Auret, and Hirst, *J.*, 1958, 221.

from the methanolysis of the reduced methylated polysaccharides formed by treatment of the methylated acidic polysaccharide with lithium aluminium hydride. An example

TABLE 3.

Examination of methanolysis products from methylated gum ghatti by gas chromatography.

| Sugars previously characterised | Approx. relative propn. | Relative retention times ($T$) of methyl glycosides: Column *a* | Column *b* |
|---|---|---|---|
| 2,3,4-Tri-*O*-methylrhamnose | + | 0·45 — | (0·46) — |
| 2,3,5-Tri-*O*-methylarabinose | ++++ | 0·56, 0·72 | 0·46, 0·59 |
| 2,3-Di-*O*-methylarabinose | ++ | 1·56, (1·76) | (0·64), (0·84) |
| | | (1·93) — | 0·96 — |
| 2,4-Di-*O*-methylarabinose | ++ | 2·23, 2·34 | 1·07, 1·11 |
| 2,5-Di-*O*-methylarabinose | ++ | 1·88, 3·44 | 0·70, (1·03) |
| 2,3,4,6-Tetra-*O*-methylgalactose | + | (1·80) — | 1·60 — |
| 2,3,4-Tri-*O*-methylgalactose | +++ | 7·5 — | 2·60, 2·89 |
| 2,4-Di-*O*-methylgalactose | +++ | — — | 3·72, 4·40 |
| 2,3,4-Tri-*O*-methylglucuronic acid | + | (2·52), 3·25 | 1·76, 2·22 |
| 2,3-Di-*O*-methylglucuronic acid | ++ | 8·4, 9·3 | Not detected |
| Sugar not previously recognised | | | |
| 2,4,6-Tri-*O*-methylgalactose | + | 4·16, 4·75 | 2·08, 2·35 |

of the use of this procedure is given in the accompanying paper on *Acacia senegal* gum (gum arabic).[15]

## EXPERIMENTAL

Gas–liquid partition chromatography was carried out by using a " Pye Argon Chromatograph " according to the procedure of Bishop and Cooper.[2] Separations were made on the following columns (120 × 0·5 cm.) at gas flow rates of 80—100 ml./min.: (*a*) 15% by weight of butan-1,4-diol succinate polyester [2] on acid-washed Celite (80—100 mesh) at 175°, (*b*) 10% by weight of polyphenyl ether [*m*-bis-(*m*-phenoxyphenoxy)benzene] on acid-washed Celite at 200°.

Methyl glycosides were formed by refluxing the sugar with methanolic 3% hydrogen chloride for *ca.* 6 hr. Longer reaction times (*ca.* 18—24 hr.) were employed during the methanolyses of methylated polysaccharides in order to achieve adequate depolymerisation.

The author thanks Professor E. L. Hirst for his interest and advice, and Drs. J. Baillie, V. P. Bhavanandan, D. A. Rees, K. M. Ross, and T. M. Wood, and Messrs. Nasir-ud-din, J. L. Whyte, and R. Young for assistance in the preparation of samples.

DEPARTMENT OF CHEMISTRY,
UNIVERSITY OF EDINBURGH. [*Received, August 30th*, 1962.]

[15] Aspinall, Charlson, Hirst, and Young, *J.*, 1963, 1696.