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QUANTITATIVE ESTIMATION OF NEUTRAL SUGARS BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

This paper reports an improved method for separating by isothermal gas–liquid chromatography, the hexoses, the pentoses, five deoxy sugars associated with natural products, and erythrose, as their alditol acetates. The solvent peak shows virtually no tailing, thus making quantitative estimation of the pentose sugars as accurate as that of the hexoses. It is also shown that the surface characteristics of the solid support to a large extent determine the degree of resolution of the various sugars. The conditions described give sharp, well resolved, and relatively narrow peaks for the slower moving components, thus improving the accuracy with which they can be measured quantitatively.

INTRODUCTION

During studies at present being undertaken in this laboratory, it became necessary to separate on a quantitative basis a wide range of hexose and pentose sugars. Because of the number of peaks which would have been produced by using the trimethylsilyl ethers of the parent carbohydrates, we chose to work with the O-acetyl glycitols[1,2]. Although these derivatives will not distinguish between certain pairs of sugars which give a common alditol (lyxose/arabinose, gulose/glucose, and altrose/talose), they do give only one peak for each sugar, and adequate separation of the peaks representing each pair.

Recently, various workers have reported the separation of a range of alditol acetates by GLC[3–8]. In most cases it was necessary to use either a programmed temperature gradient, or alternatively, a carrier gas flow rate such that glucitol acetate was eluted from the column only after a long time, resulting in flat peaks which were difficult to measure quantitatively. We have tried to impose conditions which are easily duplicated and give a rapid result, with good resolution between successive components. To this end we have worked isothermally, and stipulated the maximum retention time of hexa-O-acetyl glucitol to be 35 min. KIM *et al.*[7], using ECNSS-M on Gas-Chrom Q, have used these parameters, but have studied only four sugars, of which fucose was eluted with the tail of the solvent peak, thus making accurate measurement difficult.

EXPERIMENTAL

Chromatography

Analytical gas chromatography was carried out on a Pye Series 104 Model 64B gas chromatograph, used as a single column instrument, and fitted with a hydrogen flame detector. The sensitivity of the gas chromatograph was adjusted so that 1×10^{-9} A gave a full-scale deflection (1 mV) on the recorder. Columns were glass, of 9 ft. (2.74 m) length (unless otherwise indicated) by 0.25 in. (6.33 mm) O.D. and 0.125 in. (3.16 mm) I.D.; injections were made 1 cm into the solid support, which was heated through the first 2.5 cm by the injector heater. The area under each curve was measured with a Disc integrator (Model 201-B) which was attached to the recorder. Chromatography was carried out isothermally at 199° for the columns used except where other temperatures are indicated, with the detector oven and injection port at 250°. The optimum flow rate of the nitrogen carrier gas was calculated from a VAN DEEMTER plot[9] of flow rate *versus* HETP using penta-O-acetyl xylitol for calibration. The flow rate for the maximum number of theoretical plates was found to be 40 ml/min (measured at 23°), and this was used for all the column packings tested. Hydrogen and air flow rates were set at 50 ml/min and 600 ml/min respectively.

Materials

The parent carbohydrates or their derivatives were obtained from the following sources:

(a) Galactose, glucose, xylose, arabinose, mannose, rhamnose, ribose (British Drug Houses, Toronto).

(b) Fucose, 2-deoxy-galactose, 2-deoxy-glucose, 2-deoxy-ribose (Pierce Chemical Co., Rockford, Ill.).

(c) Erythritol (Pfanstiehl Laboratories Inc., Waukegan, Ill.).

(d) Allose prepared by a modification of the method of SOWA *et al.*[10].

(e) A syrupy mixture of idonolactone and gulonolactone by the KILIANI[11] cyanohydrin synthesis on xylose.

(f) Altronolactone by the KILIANI cyanohydrin synthesis on ribose, and deionization of the calcium altronate separated from the reaction mixture[12].

EGSS-Y, EGSP-Z and three different lots of EGSS-X were obtained from Applied Science Laboratories, State College, Pa., as was Gas-Chrom Q. Neopentylglycol sebacate, Chromosorb P, and the various grades of Chromosorb W were supplied by Chromatographic Specialties, Brockville, Ontario.

Column packings

The columns and packing materials examined were as follows (liquid phase as % w/w of support):

(a) 10 % neopentylglycol sebacate polyester on acid-washed DMCS-treated Chromosorb W[13] 60–80 mesh [a 5 ft. (1.52 m) column at 205°];

(b) 3 % neopentylglycol sebacate polyester on acid-washed DMCS-treated Chromosorb W 60–80 mesh [a 5 ft. (1.52 m) column at 205°];

(c) 3 % EGSS-X on Gas-Chrom Q 100–120 mesh;

(d) 2 % EGSS-X (Lot 124) on non-acid-washed Chromosorb W 60–80 mesh;

(e) 2 % EGSS-X (Lot 159) on non-acid-washed Chromosorb W 60–80 mesh;

(f) 2 % EGSS-X (Lot 160) on non-acid-washed Chromosorb W 60–80 mesh;
(g) 2 % EGSS-X (Lot 159) on DMCS-treated Chromosorb W 70–80 mesh;
(h) 2 % EGSS-X (Lot 159) on Gas-Chrom Q 60–80 mesh;
(i) as (c) but length of column 7 ft. (2.13 m).

Preparation of samples

In quantitative studies the sugars were compared with glucose on a molar basis. Standard solutions of all the sugars except idose, altrose, and erythrose were made up to a concentration of 0.01 *M*. Reduction and acetylation of the sugar mixtures was achieved by using a modification[14] of a method reported by ONISHI *et al.*[13]. The solution of sugar to be studied (5 ml) was added to the solution of glucose (5 ml) and reduced for 1 h with sodium borohydride (40 mg). Excess sodium borohydride was destroyed by the addition of a few drops of glacial acetic acid, and the solution was evaporated to a syrup. Three portions of 1 % methanolic hydrogen chloride (3 ml) were added and evaporated off under reduced pressure (20 mm Hg, 40°) to remove the borate as methyl borate. The dry powder left after the evaporation was dissolved in acetic anhydride (5 ml) to which had been added concentrated sulphuric acid (0.12 ml), and heated on a water bath (80° for 1 h). The acetylated alditols were then recovered by pouring the cooled solution into a previously frozen mixture of pyridine (5 ml) and water (10 ml). After 10 min the resulting mixture was extracted with chloroform, the chloroform extract then being washed successively with water, 1 *N* H_2SO_4, 5 % $NaHCO_3$, and water, and finally dried over anhydrous sodium sulphate. The chloroform extract was then evaporated to an amber syrup, dissolved in dry chloroform (4 ml), and applied to the gas chromatograph as a 1 μl sample. Idose and altrose, which were used as their lactones, were not included in the quantitative study, but were reduced with sodium borohydride at alkaline pH directly to iditol and altritol, and then acetylated in the same manner as the aldoses. Erythritol was mixed with an equal molar amount of glucitol and acetylated directly. The retention times (calculated by measuring the distance of the peak top from the injection point) of the sugars, relative to hexa-O-acetyl glucitol, were measured concurrently by reducing and acetylating a mixture containing 1 ml of each of the twelve 0.01 *M* solutions.

RESULTS AND DISCUSSION

One of the major difficulties in the assay of the sugars from polysaccharide hydrolysates by GLC has been in the separation of the alditol acetates (particularly glucitol and galactitol acetates) such that sharp peaks and maximum resolution are obtained, to facilitate accurate measurement of the peak areas. In view of this, we have calculated the efficiency of the columns used by measuring the resolution between glucitol and galactitol acetates according to the formula $R = 2d/W_1 + W_2$, where d is the distance between the peaks, and W_1 and W_2 are the lengths of baseline cut by the tangents to the two peaks[15]. Resolution is approximately 98 % when $R = 1.0$, and 99.7 % when $R = 1.5$. In addition to the acetates of glucitol and galactitol, it was found that the acetates of the pairs rhamnitol/fucitol, and arabitol/ribitol were not completely separated. Altritol and mannitol acetates could not be separated on any of the columns included in this study. These two sugars, however, seldom appear together in natural products. The acetates of 2-deoxy-glucitol and 2-deoxy-galactitol,

which have been subjected to GLC on a complex liquid phase without being separated[8], were slightly resolved on one of the columns used.

The resolution of the pairs of acetates glucitol/galactitol, rhamnitol/fucitol, and ribitol/arabitol was calculated for each of these columns, and the time for the elution of glucitol acetate was noted (Table I). These results were not significantly different when performed on duplicate columns nor when columns d, e, and f were made up using a different batch of Chromosorb W.

TABLE I

RESOLUTION OF THREE PAIRS OF ALDITOL ACETATES USING DIFFERENT COMBINATIONS OF LIQUID PHASE AND SOLID SUPPORT

Column	*R(rhamnitol/ fucitol)*	*R(ribitol/ arabitol)*	*R(glucitol/ galactitol)*	*Time of glucitol (min)*
a	0.90	1.15	[a]	79
b	0.32	0.64	[a]	24
c	2.09	2.17	1.07	80
d	1.20	1.49	1.06	33
e	1.03	1.37	0.949	30
f	1.36	1.46	0.976	29
g	1.36	1.32	0.545	28
h	1.38	1.40	0.565	36
i	1.74	1.85	0.88	40

[a] No separation.

Attempts to separate these pairs of alditol acetates using liquid loadings of 1% EGSS-Y, 3% EGSP-Z, and 10.5% EGSS-X on Chromosorb P 100–200 mesh were unsuccessful; the best separation of glucitol and galactitol acetates with an *R* value of 0.405 was obtained with the 1% EGSS-Y column. A marked change in the polarity of the column occurred when a liquid loading (EGSS-X) of 10.5% instead of 2% was used. In this case, it was noted that the peaks tended to be poorly resolved within one class of sugar (*i.e.* pentoses, hexoses, 6-deoxy-hexoses) with large retention volumes between the different classes. Similar changes in polarity have been observed when methyl esters of fatty acids are subject to gas chromatography on EGSS-X[16]. Column "c" appeared to be the column of choice, except for the long retention time (80 min) of hexa-O-acetyl glucitol, though column "d", with a much shorter retention time, gave a resolution of better than 98% for all three of the pairs. An attempt to shorten the retention times on column "c" by shortening the column (column "i") made the resolution of the glucitol/galactitol pair unacceptable. The use of EGSS-X at a loading of 2% on non-acid-washed Chromosorb W does, however, show a slight dependence on the batch of polymer used (columns "d", "e", and "f"), though all three columns gave substantially better results than any other column tested. An interesting point which emerges from the testing of the columns was that the activity of the solid support material played a significant part in the resolution of the glucitol/galactitol pair; the best resolution being obtained on the most active support, which was Chromosorb W (ref. 17) without prior deactivation treatment (Table II).

The foregoing experimental work indicated that of those tested, the column of choice for quantitative analysis of the wide range of glycoses encountered in natural

TABLE II

EFFECT OF SOLID SUPPORT ON THE RESOLUTION OF HEXA-O-ACETYL GLUCITOL AND HEXA-O-ACETYL GALACTITOL USING 2% EGSS-X AS THE LIQUID PHASE

Solid support	*R(glucitol/galactitol)*
Non-acid-washed Chromosorb W	1.06
DMCS treated Chromosorb W	0.545
Gas-Chrom Q	0.565

products would be 2% EGSS-X on non-acid-washed Chromosorb W used at 199° with a nitrogen carrier gas flow rate of 40 ml/min. An increase or decrease of 5° in the temperature of the column decreased the resolution of the three sugar pairs, as did any gross change in the carrier gas flow rate. The relative retention times of fifteen glycitol acetates, and the relative molar response of thirteen of these acetates were measured on this column.

Relative retention time

The retention times of the acetates relative to hexa-O-acetyl glucitol, were measured on the column as a mixture of equimolar proportions of twelve of the sugars (Fig. 1). Iditol, altritol, and erythritol acetates were measured separately, again using hexa-O-acetyl glucitol as the standard. The observed relative retention times are given in Table III.

Molar response

It has been stated[1] that on-column injection of the alditol acetates prevents their decomposition during passage through the packing material. Recently, however,

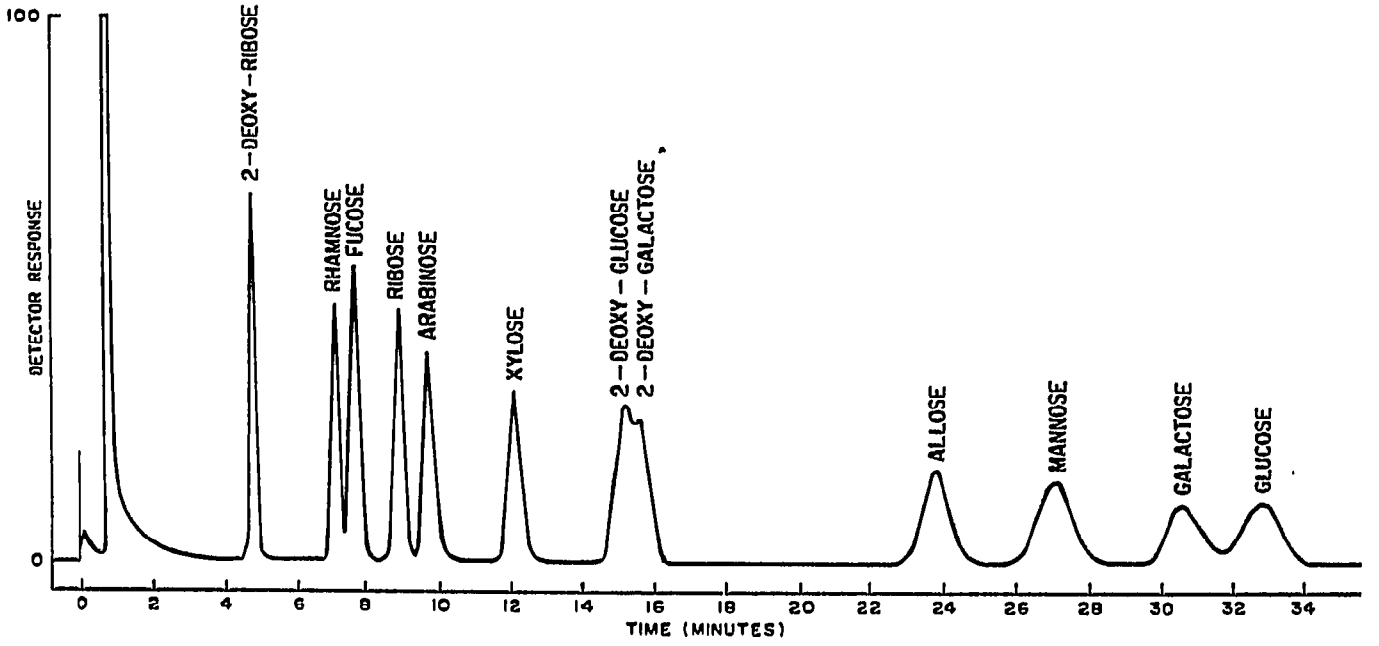

Fig. 1. Gas–liquid chromatogram of the alditol acetates from a standard 0.01 *M* mixture of the parent carbohydrates.

TABLE III

RELATIVE RETENTIONS FOR THE ALDITOL ACETATES USING HEXA-O-ACETYL GLUCITOL AS THE STANDARD

Parent carbohydrate	*R.T. (rel. hexa-O-acetyl glucitol)*
Erythrose	0.131
2-Deoxy-ribose	0.146
Rhamnose	0.221
Fucose	0.235
Ribose	0.272
Arabinose	0.297
Xylose	0.370
2-Deoxy-glucose	0.463
2-Deoxy-galactose	0.475
Allose	0.728
Mannose	0.840
Altrose	0.842
Galactose	0.930
Glucose	1.000
Idose	1.142

JANSEN *et al.*[18] using ^{14}C-labelled trimethylsilyl sugars have shown that only one fourth of the glucose derivative is eluted from the column. As far as can be ascertained, no similar study has been performed with the alditol acetates.

This paper uses the term relative molar response to be the flame ionization detector (F.I.D.) response after chemical reaction of one mole of the sugar and passage through the column of the derivative, compared to the response of one mole of glucose treated in the same manner.

The relative molar responses of the various sugars were calculated by reducing and acetylating equimolar mixtures of glucose and the sugar whose response was being measured. With erythritol, the pentoses, the 6-deoxy-hexoses, and 2-deoxy-ribose, it was found essential to remove the last traces of pyridine from the acetylated glycitols in order to minimize tailing of the solvent peak. In general, drying under water pump vacuum at 50° for 1 h ensured that the solvent peak returned to the baseline within 2 min of the injection, and thus did not interfere with the first component (erythritol acetate) which was eluted at 3.2 min. The individual response factors were calculated relative to hexa-O-acetyl glucitol by using the equation:

$$\text{response} = \frac{\text{area of x}}{\text{area of glucitol}}$$

Duplicate measurements (a and b) were made by two separate injections of a common solution. The chemical reproducibility of the method was checked by combining equimolar amounts of all the sugars (except erythritol), reducing and acetylating the mixture, and measuring the response of the alditol acetates from the chromatogram shown in Fig. 1. For each sugar pair, the area under the curve for peaks not completely resolved was calculated by dividing the total integrator counts at the point of valley inflection. The results for the single determinations and that on a mixture are given in Table IV.

TABLE IV

RELATIVE RESPONSE FACTORS OF THE ALDITOL ACETATES (FOR THE ANALYSIS FROM SUGAR THROUGH TO GLC) ON A MOLAR BASIS USING HEXA-O-ACETYL GLUCITOL AS THE STANDARD

Parent carbohydrates	*Relative response factor (single sugar)*		*Relative response factor (mixture)*
	a	*b*	
Erythrose	0.76	0.78	[a]
2-Deoxy-ribose	0.82	0.79	0.80
Rhamnose	0.80	0.80	0.80
Fucose	0.91	0.90	0.90
Ribose	0.95	0.94	0.97
Arabinose	0.94	0.92	0.91
Xylose	0.90	0.91	0.89
2-Deoxy-glucose	1.06	1.04	0.89[b]
2-Deoxy-galactose	1.04	1.04	0.87[b]
Allose	1.01	1.02	0.99
Mannose	1.08	1.08	1.07
Galactose	0.98	0.99	0.99
Glucose	1.00	1.00	1.00

[a] Not included in the mixture.
[b] Due to the low degree of resolution of this pair, even under ideal conditions, it would be expected that quantitative measurements taken on a mixture of the two would be inaccurate.

The column that has been described was used for approximately 2000 h at 199° before any noticeable change in the resolution of the peaks was observed. Under the conditions given, we have avoided the use of a complex liquid phase and programmed temperatures as used by LEHNHARDT *et al.*[8] and have widely extended the range of sugars and eliminated the interference of the solvent peak with the first sugars eluted as reported by KIM *et al.*[7]. The only sugars not more than 98 % resolved were altrose and mannose, which were not resolved at all, and 2-deoxy-glucose and 2-deoxy-galactose, which, subject to the column being carefully packed, were partially separated.

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REFERENCES

1 S. W. GUNNER, J. K. N. JONES AND M. B. PERRY, *Can. J. Chem.*, 39 (1961) 1892.
2 S. W. GUNNER, J. K. N. JONES AND M. B. PERRY, *Chem. Ind. (London), Ser. A*, (1961) 255.
3 J. S. SAWARDEKER, J. H. SLONEKER AND A. JEANES, *Anal. Chem.*, 37 (1965) 1602.
4 E. SJÖSTRÖM, P. HAGLUND AND J. JANSON, *Svensk Papperstid.*, 69 (1966) 381.
5 E. P. CROWELL AND B. B. BURNETT, *Anal. Chem.*, 39 (1967) 123.
6 J. M. OADES, *J. Chromatog.*, 28 (1967) 246.
7 J. H. KIM, B. SHOME, T.-H. LIAO AND J. G. PIERCE, *Anal. Biochem.*, 20 (1967) 258.
8 W. F. LEHNHARDT AND R. J. WINZLER, *J. Chromatog.*, 34 (1968) 471.

9 J. J. VAN DEEMTER, F. J. ZUIDERWEG AND A. KLINKENBERG, *Chem. Eng. Sci.*, 5 (1956) 271.
10 W. SOWA AND G. H. S. THOMAS, *Can. J. Chem.*, 44 (1966) 836.
11 H. KILIANI, *Ber.*, 19 (1886) 767.
12 F. L. HUMOLLER, in R. L. WHISTLER AND M. L. WOLFROM (Editors), *Methods in Carbohydrate Chemistry*, Vol. 1, Academic Press, New York, 1962, p. 102.
13 H. ONISHI AND M. B. PERRY, *Can. J. Microbiol.*, 11 (1965) 929.
14 J. K. N. JONES, personal communication.
15 L. S. ETTRE, *J. Gas Chromatog.*, 1 (1963) 36.
16 R. G. ACKMAN, *Lipids*, 2 (1967) 502.
17 J. F. PALFRAMAN AND E. A. WALKER, *Analyst*, 92 (1967) 71.
18 E. F. JANSEN AND N. C. BAGLAN, *J. Chromatog.*, 38 (1968) 18.