

530. *The Quantitative Analysis of Mixtures of 1:3:4:6-Tetramethyl, 1:3:4-Trimethyl, and 3:4-Dimethyl Fructoses by Partition Chromatography.*

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A small-scale analytical separation of the three sugars mentioned in the title is described. Owing to the volatile and hygroscopic nature of the tetramethyl sugar, recourse is made to a colorimetric method for its determination in the appropriate chromatographic fraction. The especial value of paper chromatography for the characterisation of the sugars is emphasised.

THE analysis of mixtures of methylated derivatives of D-fructose has not hitherto been advanced to the stage of accuracy now obtaining in the glucose series, despite the many problems presented by the widespread occurrence of fructosans in the plant and microbiological kingdoms. The early work of the St. Andrews school has been continued mainly at Birmingham and at Hamburg; the analytical large-scale technique employed has usually been based on fractional distillation of the different methylfructosides. Such methods require large amounts of material and they suffer markedly from scarcity of data whereby to characterise the components of the fractions.

To facilitate such analyses we endeavoured to apply the partition-column procedure described by one of us (Bell, *J.*, 1944, 473; 1948, 992) for the separation of the methylated glucoses, and have met with some success. By partition chromatography we have achieved the complete separation and quantitative recoveries of 1:3:4:6-tetramethyl, 1:3:4-trimethyl, and 3:4-dimethyl D-fructose from mixtures of all three, working over a range of 100—300 mg. of each sugar. Unreported experiments have shown that up to 1500 mg. of a sugar can be successfully handled. We also attempted experiments using 3:4:6-trimethyl D-fructose, but difficulties of obtaining this sugar in a pure state have hindered successful analyses in this instance.

Our new method differs in two main respects from the above-mentioned analysis of methylated glucoses. First, as found in this laboratory by Dr. G. D. Greville (1944, unpublished work) the partition ratios of the methylated fructoses between water and most ordinary immiscible solvents differ so greatly from those of their glucose isomers that a separate series of partitioning solvents has to be used. Secondly 1:3:4:6-tetramethyl fructose is noticeably volatile at room temperatures at the vacuum of the water-pump. It is therefore impossible quantitatively to recover this sugar in the dry state. Considering the tetramethyl and trimethyl fructoses we observed that toluene was satisfactory as the mobile phase of the partition column. There is a greater difference between the partition values of the two sugars between water and toluene than with any other convenient solvent tried. Owing probably to adsorption by the silica, it was found necessary to add a small quantity (0.33% v/v) of ethanol to the toluene used for elution.

Small-scale columns (Bell, *loc. cit.*) prepared with ethanolic toluene were used for trial experiments. At various stages of development, the columns were extruded and, after drying at 110°, the position of the bands occupied by fructose derivatives discovered by streaking with hot (*ca.* 90°) 0.2% orcinol in 66% sulphuric acid. Unpublished work by Dr. J. S. D. Bacon in

this laboratory has shown that a reddish-brown colour develops very rapidly in the regions containing free or combined ketoses. Aldoses, on the other hand, react much more slowly and develop a red to purple tint. Experiments showed that, after complete elution of the tetramethyl sugar, the tri- and the di-methyl fructoses remained at the top of the column. The trimethyl sugar could then be eluted by chloroform containing 5% (v/v) of *n*-butanol, still leaving the dimethyl fructose at the top of the column. The last sugar was recovered by extraction of the column by methanol.

When we examined the recovery of 1 : 3 : 4 : 6-tetramethyl fructose, using the gravimetric assay described for the glucose series, we encountered the above-mentioned volatility of the sugar. Low yields, progressive decreases in weight of samples dried in a vacuum, and browning of the sodium hydroxide used as the drying agent convinced us that losses due to evaporation were taking place. A highly purified sample of the tetramethyl sugar, after a final distillation (bath temp. 50—60°/0.01 mm.) in an apparatus of the type used by Ellis (*Chem. and Ind.*, 1934, 77) was examined with the following results :

Period of drying at 15 mm. (hrs.)	0	1	2	2	17	5	20	48
Wt. of sugar (mg.)	700.1	679.2	677.2	676.2	674.0	673.2	672.2	670.2
Loss in weight (mg.)	—	20.9	2.0	1.0	2.2	0.8	1.0	2.0

During each period, browning of the sodium hydroxide in the desiccator was again noted. The large decrease in weight during the first drying period is probably due to loss of water vapour (see p. 2524).

Having shown that gravimetric assay of 1 : 3 : 4 : 6-tetramethyl fructose is inaccurate, we next examined the possibility of determining the sugar in a dilute aqueous solution by a colorimetric method. This proved to be readily done and gave excellent recoveries. It also proved possible to characterise the sugar of this fraction as being 1 : 3 : 4 : 6-tetramethyl D-fructose uncontaminated by lower homologues. (It must be emphasised that gravimetric assay of 1 : 3 : 4-trimethyl and 3 : 4-dimethyl fructoses presented no difficulties.) The tetramethyl sugar is not volatile in steam or in the vapours of organic solvents provided the solutions be not too concentrated. The procedure finally adopted for analysis of the mixed sugars is as follows : An aqueous solution, representing the neutralised acid hydrolysate of a methylated fructosan, is extracted ten times with chloroform. The combined extracts are concentrated to about 50 ml., and the chloroform displaced by toluene by distillation at low temperature to yield a final volume of about 50 ml. This solution is quantitatively pipetted on to the surface of a 12-g. silica column. The tetramethyl fraction is then eluted with toluene containing 0.33% (v/v) of ethanol. The eluate is concentrated to about 50 ml. as before and the 1 : 3 : 4 : 6-tetramethyl fructose partitioned back into water and determined colorimetrically by the method of Cole, Hanes, Jackson, and Loughman (in the press).

The original aqueous phase containing the "tri-" and "di-"fractions is evaporated to dryness, and the residue is dissolved in chloroform and transferred quantitatively to the partition column. The total trimethyl sugar is then eluted by chloroform containing 5% (v/v) of *n*-butanol and determined gravimetrically. The dimethyl fructose left on the column is also determined gravimetrically after elution by methanol.

The characterisation of the three sugars obtained from the column separations offered some difficulty, especially with 1 : 3 : 4 : 6-tetramethyl fructose. Small-scale operations demand measurements on small amounts of material which readily take up moisture, the sugars all being very hygroscopic. For 1 : 3 : 4 : 6-tetramethyl fructose we have adopted the procedure of subjecting the sugar to a preliminary distillation in a high vacuum, using an apparatus similar to that described by Ellis (*loc. cit.*). In this way the material is obtained reasonably free from moisture and may be used directly for measurements of specific rotation and refractive index. For methoxyl determinations, however, it was found necessary to subject the sample for analysis to a second drying in high vacuum over phosphoric oxide (see p. 2524); 3 : 4-dimethyl fructose is a very viscous syrup and we do not consider its refractive index a very satisfactory means of characterisation owing to the difficulty of determining the value for the pure dry sugar. Each of the "tetra-", "tri-", and "di-"methyl fractions obtained by elution was examined by the paper chromatogram (Hirst, Hough, and Jones, this vol., p. 928). In no instance was there any evidence of the presence of any substance other than the expected sugar.

EXPERIMENTAL.

Obligatory Precautions.—The precautions described by Bell (*J.*, 1944, 473) were observed throughout. Ethanol and methanol were distilled, in all-glass apparatus, from sodium hydroxide and silver oxide. As in the previous paper, water-immiscible solvents for partition were equilibrated with water before

use. Solvents were evaporated under reduced pressure, the temperature of the water-bath being kept below 30°. Separating-funnel partitions were done between equal volumes of the two phases.

Silica gel. This was prepared according to Tristram's modification (*Biochem. J.*, 1946, **40**, 723) of the original procedure of Gordon, Martin, and Syngé (*ibid.*, 1943, **37**, 80) in that one volume of water-glass was diluted initially with one volume each of water and ice. The silica was finally dried at 110° for 24 hours.

Preparation of the column. One part of silica, finely ground in a mortar, was mixed with one-half its weight of water, and grinding continued to give a fine dry powder. This powder was then stirred with sufficient ethanolic toluene (EtOH, 0.33% v/v) to give a slurry which was then poured into a suitable glass tube fitted at the lower end with a porcelain disc and cotton-wool plug. When the column of gel had settled it was de-greased by passage of three column-lengths of ethanolic toluene.

Recovery Experiments on Artificial Mixtures.—The columns used were prepared from 12 g. of silica and were held in tubes 35 mm. in diameter and about 1 m. long. 5 ml. of toluene solutions containing known amounts of the sugars (100–200 mg. of each of 1 : 3 : 4 : 6-tetramethyl and 1 : 3 : 4-trimethyl fructose) were pipetted on to the surface of the silica and allowed to sink into the column. One column-length of ethanolic toluene was carefully added, and collection of the eluate commenced. A further 15 column-lengths of eluting solvent were then passed. The total eluate was concentrated, in presence of a little barium carbonate, to a volume of about 50 ml. and extracted 18 times with water. The combined aqueous phases were filtered into a 1-l. graduated flask and made up to the mark with washings of the filter-paper. The tetramethyl fructose in aliquots of 2.0 ml. was determined as stated on p. 2523, for the specific estimation of ketoses. Typical recoveries of tetramethyl fructose are shown below.

Amount of Me ₄ sugar put on column (mg.)	109.0	109.0
Sugar found by colorimetric determination (mg.)	107.0	107.5
Recovery (%)	98	98

On to the toluene-washed column were pipetted 5 ml. of a chloroform solution of 3 : 4-dimethyl fructose containing 100–200 mg. of the sugar. (The dimethyl fructose is not soluble in toluene). The column was then treated with 8 column-lengths of chloroform containing 5% (v/v) of *n*-butanol. The total eluate was evaporated to dryness under reduced pressure, in presence of a little barium carbonate, water being added to assist removal of the butanol. The dried residue was extracted with dry ether, and this solution evaporated in a slight modification of Bell's apparatus (*loc. cit.*). The temperature of the air-bath for this operation must be kept below 30° or decomposition of the sugar results. On seeding and drying to constant weight in a high vacuum over phosphoric oxide and sodium hydroxide, the 1 : 3 : 4-trimethyl fructose set to a hard mass of needles. Typical recoveries were:

Amount of Me ₃ sugar put on column (mg.)	101	106	106	113	212
Sugar found by gravimetric method (mg.)	101	101	104	111	209
Recovery (%)	100	95	98	98	98

The chloroform–butanol-washed column was then expressed from the tube into a beaker, and the tube washed with methanol. The resulting slurry was collected on a sintered-glass funnel, and the solid washed with five lots each of 150 ml. of methanol at room temperature to avoid possible fructoside formation. The filtrate was evaporated in presence of barium carbonate, and the residue exhausted with warm, dry dichloroethylene. The resulting solution was evaporated in the evaporation apparatus at 40–50°. The sugar was finally dried to constant weight over phosphoric oxide and sodium hydroxide at about 0.05 mm. pressure. The following table contains typical recoveries.

Amount of Me ₃ sugar put on column (mg.)	100	96
Sugar found by gravimetric method (mg.)	96	92
Recovery (%)	96	96

Characterisation of 1 : 3 : 4 : 6-Tetramethyl D-Fructose.—The following measurements and n_D^{20} were used to characterise this sugar.

(a) *Specific rotation.* The literature gives the following values: +31.7° (Haworth, *J.*, 1920, **117**, 199); +31.3° (*idem*, *J.*, 1927, 1519); +25.9° (Schlubach, Knoop, and Liu, *Annalen*, 1933, **504**, 30); +32.8° (Leitch, *J.*, 1927, 588); +31.7° (Haworth, Hirst, and Ruell, *J.*, 1923, **123**, 3130); +32.9°, +31.7° (Irvine and Steele, *J.*, 1920, **117**, 1478); +30.6° (Hibbert, Tipson, and Brauns, *Canadian J. Res.*, 1931, **4**, 221). When handling dried material of the order of 0.75–1.5 g. and weighing it on the ordinary analytical balance, rotations of +31° were in fact found; but when weighings had to be from small amounts, the sugar apparently absorbed about 10% of moisture during the operation and low values for $[\alpha]_D$ tended to be obtained.

(b) R_F Value (see Hirst, Hough, and Jones, this vol., p. 938). This, in *n*-butanol–water was 0.8 for authentic material and for all column samples examined. (As our chromatograms were not controlled for temperature, figures for R_F values are given only to the first place of decimals. Controls with authentic specimens were always run simultaneously.)

(c) *Methoxyl content.* The values for the methoxyl content of 1 : 3 : 4 : 6-tetramethyl fructose recorded in the literature are sometimes lower than the theoretical value of 52.5%: e.g., 49% (Haworth, *loc. cit.*); 53.2% (Irvine and Steele, *loc. cit.*), 50.7% (Schlubach and Boe, *Annalen*, 1937, **532**, 191), 53.4% (Schlubach and Peitzer, *ibid.*, 1937, **530**, 120), 52.0% (Schlubach and Loop, *ibid.*, 1936, **523**, 130), 52.0% (Schlubach and Bandmann, *ibid.*, 1939, **540**, 285). Owing to the hygroscopic nature of the sugar we obtained low values by the micro-technique; if, however, the specimen of the sugar was first roughly measured into a small tared vessel fitted with a stopper and the whole dried over phosphoric oxide in a high vacuum (in which some of the sugar volatilises) before the final weighing was made, better values were obtained.

Characterisation of 1 : 3 : 4-Trimethyl D-Fructose.—None of our specimens crystallised without seeding. This was first made possible by a crystalline specimen kindly provided by Professor S. Peat,

F.R.S. The following measurements were used to characterise the sugar. The melting point of the untilted material tended to be unsharp (about 73°) and was not used as a means of characterisation.

(a) *Specific rotation.* Using a 5% solution of thoroughly dried pure 1 : 3 : 4-trimethyl D-fructose, we obtained the following values on keeping at 16.5°: -56.9° (60 minutes); -60.8 (19 hours); -61.6° (const.) (68 hours). This sugar is also somewhat hygroscopic, and slightly lower values were found for column specimens where the micro-balance was employed (see following table).

The literature gives the following values: -52° (at 18°, *c*, 5) (Challinor, Haworth, and Hirst, *J.*, 1934, 676), -47.6° (*c*, 1) (Schlubach and Sinh, *Annalen*, 1940, 544, 101). It should be noted that the constants quoted by Elsner ("Kurzes Handbuch der Kohlenhydrate," Leipzig, 1935, p. 373) and by Micheel ("Chemie der Zucker und Polysaccharide," Leipzig, 1939, p. 98) do not refer, as stated, to 1 : 3 : 4-trimethyl D-fructose (cf. also Hudson, "Advances in Carbohydrate Chemistry," 1946, 2, 20).

(b) *Refractive index.* The few values quoted in the literature are rarely accompanied by the temperature at which the measurement was made. The fused crystalline sugar had n_D^{20} 1.4660 (decrement for rise of 1.0°, 0.00036) and the temperature- n_D relationship followed a straight line between 15° and 40°. Hibbert, Tipson, and Brauns (*loc. cit.*, 1931, 4, 221) give n_D^{20} 1.4661 for the sugar in the liquid form (see following table).

(c) R_F Value. For pure 1 : 3 : 4-trimethyl fructose and for all specimens examined R_F was 0.60.

(d) *Methoxyl content.* No marked difficulty was found in determining this. Pure material showed OMe, 42.2% (calc.: 41.9%) (see following table).

(e) *Formaldehyde production after periodate oxidation.* Bell, Palmer, and Johns (this vol., p. 1536) have shown that 1 mole of 1 : 3 : 4-trimethyl fructose yields 1 mole of formaldehyde when oxidised by sodium metaperiodate in phosphate buffer of pH 7.5. This fact affords a convenient check on the purity of the sugar, and can be used as a method of estimation.

Characterisation of 3 : 4-Dimethyl D-Fructose.—This was obtained as one of the major fractions of the hydrolysis products from methylated irisin; the proof of its constitution will be the subject of a future communication. McDonald and Jackson (*J. Res. Nat. Bur. Stand.*, 1940, 24, 181) have prepared this sugar by a synthetic route; they give $[\alpha]_D$ in water, -60.7°. Our material, exhaustively dried, was a viscous, colourless syrup having $[\alpha]_D$ in water -59.4° (*c*, 1.8), in chloroform -10.3° (*c*, 2) (Found: OMe, 29%). It appeared to be homogeneous when examined on the paper chromatogram. Measurements used to characterise the sugar are shown below and in the following table.

Refractive index. We found n_D^{20} 1.4848 for our purest sample (decrement for rise of 1.0°, 0.00035). There was a straight-line relationship between t and n_D over the range 15—35°.

R_F Value. For each specimen this was 0.4 in *n*-butanol-water.

The following table shows two examples of recovery experiments on a mixture of 1 : 3 : 4 : 6-tetramethyl, 1 : 3 : 4-trimethyl, and 3 : 4-dimethyl sugars in water, together with values obtained in the characterisation of these sugars.

Column no.	Sugar.	Wt. of sugar put on column (mg.).	Wt. of sugar recovered (mg.).	Recovery (%).
(1)	1 : 3 : 4 : 6 Me ₄ fructose	122	120	98.4
	1 : 3 : 4 Me ₃ fructose	100	94.1	94.1
	3 : 4 Me ₂ fructose	84.1	74.7	88.9
(2)	1 : 3 : 4 : 6 Me ₄ fructose	98	99	101
	1 : 3 : 4 Me ₃ fructose	324.2	309.7	95.3
	3 : 4 Me ₂ fructose	123.8	117.6	95.2

Value obtained for samples of sugars recovered from columns.

(a) 1 : 3 : 4 : 6-Tetramethyl fructose.

	Column no. :	(1)	(2)	Pure redistilled material.
$[\alpha]_D^{18}$ in water		28.1°	28.5°	30.4°
		(<i>c</i> , 1.1)	(<i>c</i> , 1.2)	(<i>c</i> , 3.4)
OMe, %		50.3	50.7	51.1
n_D^{20}		1.4504	1.4506	1.4506

(b) 1 : 3 : 4-Trimethyl fructose.

	Column no. :	(1)	(2)	Pure material recryst. from CCl ₄ .
$[\alpha]_D^{18}$ in water		-55.9°	-58.1°	-61.6°
OMe, %		40.9	43.2	42.2
n_D^{20} (fused crystals)		1.4660	1.4658	1.4660
Moles of H·CHO per mole sugar after NaIO ₄ oxidation ...		1.02	1.02	1.00

(c) 3 : 4-Dimethyl fructose.

	Column no. :	(1)	(2)	Pure material.
$[\alpha]_D^{18}$ in water		-60.1°	-58.8°	-59.4°
OMe, %		29.57	27.9	29.3
n_D^{20}		1.4842 *	1.4830 *	1.4848

* Variation in values probably due to difficulty in drying.

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