342. *Quantitative Analysis of Mixtures of Sugars by the Method of Partition Chromatography. Part I. Standardisation of Procedure.*

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A method is described for the separation and determination of mixtures of sugars (total weight *ca.* 0·1—1·0 mg.) on the paper chromatogram. The method is accurate to within $\pm$ 2%. Polysaccharides in which the ratio of sugars had already been determined have been examined using this new method, and the results are in agreement with those previously recorded.

The identification and determination of the components of mixtures of sugars have become a matter of great importance in the study of complex polysaccharides such as the plant gums and mucilages. Among the most commonly occurring sugars are galactose, mannose, arabinose, and xylose, to which must be added glucose, fructose, rhamnose, and fucose, which are of less frequent occurrence. Methods for the determination of a single sugar are comparatively simple and have been widely used, but, when mixtures of sugars are to be examined, the problem becomes much more difficult.

Earlier attempts to solve this problem were based on the use of a combination of various analytical methods, the combination being chosen to suit the particular mixture under examination. In favourable circumstances a mixture of two sugars could be determined by a combination of two polarimetric equations, or combined polariscopic and reduction equations (Browne, *J. Amer. Chem. Soc.*, 1906, **28**, 439). Similarly, a mixture of three sugars could be examined by a combination of polarimetric and reduction methods and one selective method, or by two selective methods combined with a determination of total reducing power (Zerban and Wiley, *Ind. Eng. Chem. Anal.*, 1934, **6**, 354). These methods are, by their nature, of limited application.

Attention has been directed to extending the scope of the selective methods. This involves a search for a specific precipitant for each individual sugar. Thus galactose gives an insoluble phenylmethylhydrazone (van Ekenstein and Lobry de Bruyn, *Rec. Trav. chim.*, 1896, **15**, 225), mannose an insoluble phenylhydrazone (Fischer and Hirschberger, *Ber.*, 1888, **21**, 1807), and arabinose an insoluble benzoylhydrazone (Davidis, *Ber.*, 1896, **29**, 2311), although in the last case rhamnose interferes when present in considerable amounts and may, under favourable conditions, be determined as such.

With the above precipitants, the necessity for control experiments has been avoided by carrying out the determination under standard conditions (Hirst, Jones, and Woods, *J.*, 1947, 1048). By a similar technique, xylose can be determined by means of its dibenzylidene-dimethylacetal (Breddy and Jones, *J.*, 1945, 738).

In these methods the specificity of the reagent used is not always complete, *e.g.*, mannose and arabinose interfere with the determination of galactose and have to be removed or themselves determined by some other specific precipitant. The overall accuracy in this type of determination is of the order of 10% on amounts of sugar between 100 to 500 mg.

Another approach to the problem of analysing sugar mixtures is based on selective fermentation methods. It was found possible to utilise differential fermentation by two strains of yeast, of which one ferments galactose and one does not. Quantities of the order of 25—150 mg. of galactose have been determined in this way, the recovery of sugar being 92—98% (Wise and Appling, *Ind. Eng. Chem. Anal.*, 1944, **16**, 28). Similarly, xylose can be fermented with another type of yeast and from 12 to 50 mg. determined, the recovery of sugar being 96—104%. Arabinose, rhamnose, fucose, and glucuronic acid are not fermented (*idem, ibid.*, 1945, **17**, 182).

An important advance on the qualitative side was made by Partridge (*Nature*, 1946, **158**, 270), who developed an application of the paper chromatogram (Consden, Gordon, and Martin, *Biochem. J.*, 1944, **38**, 224) to the separation of individual sugars. By this technique it was possible to separate completely the components of a sugar mixture. Moreover, it was necessary to use only minute amounts of material. The present paper describes an elaboration of this technique to the determination of a sugar mixture using less than 1 mg. of material. Since the components of the mixture are completely and readily separated, a single reagent, provided it will deal with micro-quantities, serves for the determination of all the components. Such a reagent has been reported by Somogyi (*J. Biol. Chem.*, 1945, **160**, 61), and deals with amounts as small as 0·01 mg. of sugar. Quantities of about 0·1 mg. or more of each component sugar can be built up on the paper by the technique described below and subsequently analysed by the use of this reagent. The accuracy has been previously reported as $\pm$ 5% (Flood, Hirst, and Jones, *Nature*, 1947, **160**, 86 *) but with greater experience it has been found possible to work to accuracies of $\pm$ 2%.

The application of this method to the analysis of synthetic mixtures and the mixture of sugars produced on hydrolysis of polysaccharides is described below. The absolute weights of individual sugars present in the synthetic mixtures have been determined by weighing in, before analysis, a foreign sugar having an R_F value different from the sugars to be determined. This foreign sugar serves as a reference compound, and from a knowledge of the ratio of sugars present the absolute weight of each compound is readily calculated.

The polysaccharides chosen had been analysed previously by other methods. The hydrolysis of these polysaccharides was repeated on the macro-scale and small samples taken for analysis on the paper chromatogram. The results are collected in the Table.

	Mol. ratio of sugars present in the products of hydrolysis.					
Polysaccharide.	Found on the micro-scale.			Previously reported.		
	Arabinose.	Xylose.	Galactose.	L-Arabinose.	D-Xylose.	D-Galactose.
Almond-tree gum [1]...	2·07	1·0	—	2	1	—
Cherry-tree gum [2] ...	3·7	—	1·0	3·7	—	1
Cholla gum [3]	3·0	0·9	1·0 †	3	1	1 †

[1] Brown, Hirst, and Jones (previous paper).
[2] Jones, *J.*, 1939, 562.
[3] Brown, Hirst, and Jones (forthcoming publication).
† This figure does not include the galactose component of the aldobionic acid fraction. The molecular ratios quoted by Flood, Hirst, and Jones (*Nature, loc. cit.*) include this galactose residue.

Up to the present, attempts to resolve DL-mixtures of sugars by the use of an optically active alcohol such as menthol or amyl alcohol in place of butanol have been unsuccessful (see experimental section).

EXPERIMENTAL.

Apparatus.—The apparatus used was similar to that employed by Consden, Gordon, and Martin (*loc. cit.*), but in place of a drain-pipe a bell jar with a ground glass flange at the base was used. The jar measured approximately 55 × 20 cm. (diameter) and rested on a glass plate. The glass trough was supported on 4 glass legs, and a beaker served as a receptacle for the aqueous phase. The filter paper used was Whatman No. 1 and was cut to lengths of 60 cm. The width varied from 9 to 12 cm. according

* An alternative method for the adaption of Partridge's qualitative procedure to quantitative ends has been described by Hawthorne (*Nature*, 1947, **160**, 714), whose work was carried out simultaneously with ours but quite independently, the workers in the two laboratories being unaware of each others' experiments.

to the length of trough being used. The solvents and procedure used were as described by Partridge (*loc. cit.*).

Reagents.—The copper reagent was prepared as described by Somogyi (*loc. cit.*) and stored at 20—25°. The ferricyanide reagent was prepared as described by Hagedorn and Jensen (*Biochem. Z.*, 1923, **135**, 46); in addition a variation of this reagent was used having a buffer of potassium carbonate–potassium hydrogen carbonate (Hawkins, *J. Biol. Chem.*, 1929, **84**, 80).

Procedure.—The solution containing the sugars must be neutral, and the concentration of inorganic ions must be kept to a minimum in order to avoid water-logging of the paper during the subsequent chromatography (cf. Consden, Gordon, and Martin, *loc. cit.*, p. 229). The concentration is adjusted so that it is of the order of 1% with respect to the individual sugars.

A preliminary trial run is useful in indicating the concentration required for the analysis of a particular mixture. It has been found generally convenient to hydrolyse about 10 mg. of a polysaccharide with approximately 0·5 ml. of N-sulphuric acid in small sealed tubes (10 × 0·4 cm.) immersed in a boiling water-bath for the requisite time. On completion of the hydrolysis the tube is cooled and the solution centrifuged. One end of the tube is made slightly enlarged for the purpose of collecting precipitated solids (*e.g.*, barium sulphate). The tube is then opened and the acid neutralised with barium carbonate. It is advisable to add this material cautiously on the end of a very fine glass rod, the end of which is bent at right angles. The rod also serves as a stirrer. If these precautions are not taken there is a danger of excessive frothing and material may be lost. After neutralisation is complete (Congo-red) the solution is again centrifuged and the clear supernatant neutral liquid is ready for analysis.

Two pieces of filter paper are cut to suitable dimensions as described earlier, and spots of the solution are placed about 5 mm. apart along the starting line with a fine glass capillary. The spots are of the order of 0·002—0·004 ml. and must be of uniform size. They must not coalesce, and the two end spots must not be too near the edge of the paper. It is desirable that one of the end spots should be well separated from the next so that subsequently a small strip containing it can be cut from the paper sheet. The place at which this cut is to be made is indicated by a small pencil mark. In this way two paper sheets are prepared and hung on either side of the trough in the bell jar.

The chromatogram is allowed to run for about 45 hours, after which the sheets are removed and dried for 1 hour in an oven at 100°. The sheets are best handled by attaching clips to each end.

After drying, a small strip is cut from each paper sheet at the place marked and thus contains one end spot. The strip is sprayed with ammoniacal silver nitrate solution and returned to the oven for development.

A normal chromatogram having been obtained on the small strip, the position of the sugars can be located and the degree of separation observed. By matching this strip alongside the major sheet from which it was cut, areas can be cut from the latter each containing one only of the separated sugar components. This device has been justified by experiments which have shown (by spraying the whole sheet) that the separated sugars from each spot do lie on parallel horizontal lines providing that the sheet has hung vertically in the chromatographic apparatus. Finally, areas which are free from sugar and equal in size to each of the sugar-containing areas are cut from the same major sheet to serve as paper blanks. The filter paper was not specially purified before use because during the experiment it is completely washed by the solvent (cf. Consden *et al.*, *loc. cit.*, p. 226) and the blanks were always low and very consistent (see later).

The sugars having been separated, the next stage is their quantitative removal from the paper. Two methods are available. One is based on capillary siphoning and has also been used by Dent (*Biochem. J.*, 1947, **41**, 240). This method has been examined in the present work, but the following procedure is preferable since aseptic conditions can be maintained. To the lower coil of a ground glass spiral condenser is hooked a fine glass rod (diameter 1 mm.) having also a small hook at the lower end. The rod is of such a length that it projects below the end of the condenser so that the paper strip can be conveniently attached to it. A boiling tube having a ground-glass joint is attached to the lower joint of the condenser. This is marked so that it is known when it contains 5 ml. of liquid. When the apparatus is assembled, water is introduced through the top of the condenser until the boiling tube contains water to the mark. In this way allowance can be made for water hold-up in the paper strip. Provided the apparatus is completely vertical with the glass rod hanging vertically from the centre of the lower coil of the condenser and the paper hanging vertically and free within the tube, water flows down the condenser spiral, collects in a drop on the lower coil, slides down the glass rod, flushes the paper strip, and drops off the bottom into the boiling tube. This happens also during the subsequent refluxing of the water in the apparatus. The entire apparatus must be free from grease. Two extractors are used side by side, one for extracting the strip of paper containing the sugar and the other for its corresponding paper blank. The time of refluxing was fixed at 30 minutes to allow a wide margin of safety. After 30 minutes no sugar can be detected on the paper strip (ammoniacal silver nitrate).

Two 5 ml. volumes of solution are thus obtained, one serving as a blank and the other containing the sugar. They are already contained in two boiling tubes, and no further transfer of liquid is necessary during the final analysis. To complete this with the copper reagent, two further similar boiling tubes having ground glass joints are taken, and 5 ml. of water introduced into one to serve as a blank, whilst to the other is added 5 ml. of a standard aqueous solution of the sugar that is under analysis. This standard solution is made from pure recrystallised sugar that has been dried in a vacuum to constant weight. Its concentration is such that 5 ml. will contain as nearly as possible the same quantity of sugar as is present in the solution obtained from extraction of the paper strip. A little experience will indicate the required concentration, and it is soon found possible to judge this from the density of the spots on the small sprayed reference strip. In general, quantities of 20—50 mg. per l. are found suitable.

To all 4 tubes are now added 5 ml. of the copper reagent. The contents are gently mixed, and the tubes covered with small glass funnels and placed in a briskly boiling water-bath. Clamps are used for this purpose since the tubes must not be disturbed until after final acidification. After 25 minutes the hot water is sucked out of the bath with a filter pump, cold water being added at the same time until the bath temperature reaches 35°. This temperature is maintained for 10 minutes. Potassium

iodide solution (2·5%; 0·5 ml. when the copper reagent contains 5 ml. of N-potassium iodate per l.) is then carefully run down the side of each tube followed by 1·5 ml. of 2N-sulphuric acid. The latter is squirted into the tube from a small glass syringe so that the contents are instantly mixed and acidified. After acidification each tube is immediately closed by a ground glass stopper and the contents chilled in cold water. The contents of each tube are then titrated with 0·005N-sodium thiosulphate. Phenol-red (1 drop) is added to improve the end point, and a micro-burette graduated directly to 0·01 ml. is used Just before the end point 2 drops of starch solution (1%) are added. The tubes must be kept stoppered before titration, otherwise serious discrepancies arise owing to loss of iodine by volatilisation.

Four titration figures are thus obtained :

Copper reagent + 5 ml. of water = a
do. + 5 ml. of standard sugar solution = b
do. + 5 ml. of extract from paper blank = c
do. + 5 ml. of extract from paper containing sugar = d

$(a - b)$ is equivalent to the amount of copper reduced by 5 ml. of standard sugar solution, and $(c - d)$ is equivalent to the amount of copper reduced by the extract from the paper containing the sugar. If 5 ml. of standard sugar solution contains x mg. of sugar, the weight of sugar in the 5 ml. extract can be calculated by simple proportion and is $x(c - d)/(a - b)$. The "blank" due to the paper is measured by $(a - c)$, and is of the order of 0·1 ml. of 0·005N-sodium thiosulphate. This figure is quite constant and varies little from paper to paper, *e.g.*: paper (1), 0·12, 0·12, 0·10 ml.; paper (2), 0·16, 0·16 ml.; paper (3), 0·09, 0·10 ml.; paper (4), 0·14, 0·11, 0·11 ml.

The technique described ensures that both standard and sample are analysed under identical conditions and that the analysis is based solely on a sugar weight. No other reagent used need be standardised.

The analysis can also be carried out using calibration curves for each sugar. In this case the concentration of the sodium thiosulphate must be known.

After completion of the analysis for the individual sugars, a corresponding weight for each is obtained. If it is certain that the sugars analysed are the sole constituents of the mixture under examination. these weights can be converted directly into molecular ratios. If not, the following procedure is adopted. A foreign sugar known not to be present in the mixture under analysis and having a R_F value different from those of the sugars present is weighed in before chromatography to serve as a reference compound. From a knowledge of all the weight ratios the percentage of any component can then be readily calculated. Such a device is very useful when studying the hydrolysis of a polysaccharide. In this case the reference sugar is best weighed in immediately after hydrolysis and before neutralisation. Thus the reference sugar is involved in the same manipulative processes as the other sugars present.

Instead of the copper reagent, potassium ferricyanide can be used, with the same technique. It is well known (cf. Somogyi, *loc. cit.*, p. 61) that alkaline potassium ferricyanide is not so selective for sugars as is copper, and in this work it was found to be very sensitive to slight traces of grease. In order to obtain satisfactory results it is necessary to wash all apparatus before use with a solution of alkaline ferricyanide followed by much distilled water. On this account the papers must be handled with grease-free forceps and not with the fingers. For these reasons the copper reagent is to be preferred as being more convenient.

In the following analyses all chromatograms were run for 45 hours at 20° using n-butanol (40%)–ethanol (10%)–water (50%). Ammonia was added to the aqueous phase to a concentration of 1%.

Analysis of Mixtures of Reducing Sugars.—(a) *Determination of galactose, with arabinose as the reference compound.* Arabinose (10·41 mg.) was weighed into a solution containing galactose (9·58 mg.). The solution was separated on the chromatogram as an approximately 2% solution (total solids). Found by titration: galactose, 0·485 mg.; arabinose, 0·532 mg. The absolute amount of galactose in the solution is therefore 10·41 × 0·485/0·532 mg. = 9·49 mg. (99% recovery).

(b) *Analysis of a mixture of rhamnose and arabinose, with glucose as the reference compound.* Glucose (4·38 mg.) was weighed in to a solution containing rhamnose (9·60 mg.) and arabinose (5·80 mg.). The solution was separated on the chromatogram as an approximately 2% solution (total solids). Found by titration: glucose, 0·124 mg.; rhamnose, 0·261 mg.; arabinose, 0·157 mg. The absolute amounts of rhamnose and arabinose present in the original mixture are therefore, rhamnose, 4·38 × 0·261/0·124 mg. = 9·22 mg. (96% recovery); arabinose, 4·38 × 0·157/0·124 = 5·54 mg. (95·5% recovery).

(c) *Analysis of a mixture of arabinose, xylose, and rhamnose, with galactose as a reference sugar.* Galactose (210 mg.) was weighed into a solution containing arabinose (144 mg.), xylose (117 mg.), and rhamnose (200 mg.). The solution was separated on the chromatogram as an approximately 3% solution. Found by titration: galactose, 0·366 mg.; arabinose, 0·257 mg.; xylose, 0·207 mg.; rhamnose, 0·343 mg. Thus the absolute weights of sugar present in the solution are arabinose, 147 mg. (102% recovery); xylose, 119 mg. (102% recovery); rhamnose, 197 mg. (98·5% recovery).

Determination of Sugars Derived by Hydrolysis of Polysaccharides. Determination of the Molecular Ratios of the Sugars Produced.—(a) *Almond-tree gum.* The gum (5·42 g.) was kept at 98° with water (100 ml.) for 31 hours. The solution was neutralised with barium carbonate and filtered. The filtrate was concentrated under reduced pressure to a syrup which was exhaustively extracted with hot methanol. The extracts were concentrated to a syrup under reduced pressure. A portion of the syrup (3·23 g.) was hydrolysed with N-sulphuric acid (25 ml.) for 30 minutes in order to hydrolyse any oligosaccharides. The solution was neutralised with barium carbonate, filtered, and evaporated to a syrup as above. A portion of the syrup (20 mg.) was dissolved in water (1 ml.), and a small portion of it separated on the chromatogram. It was found that arabinose and xylose were present. In a quantitative experiment the portions of paper containing these sugars were found to contain respectively 0·224 mg. of arabinose and 0·108 mg. of xylose. These sugars were present therefore in the molecular ratio 2·07 : 1. Material other than free sugar was detected on the chromatogram, and from its position was either unhydrolysed material, or a uronic acid fraction, or both.

Estimation on the macro-scale indicated that the syrup contained L-arabinose (2 parts) and D-xylose (1 part) (Brown, Hirst, and Jones, *loc. cit.*).

(b) *Cherry-tree gum.* The gum (3·71 g.) was heated at 100° with N-sulphuric acid (100 ml.) for 6·25 hours, by which time the rotation had become constant. The solution was neutralised with barium carbonate, and the syrup isolated as described above. The syrup was dissolved in water, and a portion separated on the chromatogram as an approximately 2% solution (total solids). It was found that arabinose and galactose were present. In a quantitative experiment the portions of paper containing these sugars were found to contain respectively 0·763 mg. of arabinose and 0·251 mg. of galactose. These sugars were present therefore in the molecular ratio 3·7 : 1. Earlier work (Jones, *loc. cit.*) had indicated that the molecular ratio of arabinose to galactose in the hydrolysis products of the gum was 3·7 : 1.

(c) *Cholla gum.* The gum (64 mg.) was hydrolysed by boiling with N-sulphuric acid (10 ml.) for 90 minutes, by which time the rotation had become constant. The solution was neutralised with barium carbonate and the syrup isolated as before. A small portion of the syrup in water was separated on the chromatogram as an approximately 2·5% solution (total solids). Arabinose, xylose, and galactose were found to be present. In a quantitative experiment the portions of paper containing these sugars were found to contain 0·271 mg. of arabinose, 0·081 mg. of xylose, and 0·108 mg. of galactose. These sugars were present therefore in the molecular ratio 3 : 0·9 : 1.

Examination of the gum by macro-methods (Brown, Hirst, and Jones, *loc. cit.*) indicated that the hydrolysis products included L-arabinose (3 parts), D-xylose (1 part), and D-galactose (1 part), in good agreement with the chromatogram results.

Attempted Resolution of DL-*Arabinose* (with W. G. C. FORSYTH).—An attempt was made to resolve DL-arabinose (1% aqueous solution) with (*a*) *lævo*menthol, and (*b*) *lævo*amyl alcohol, in place of butanol as the organic phase in the chromatogram. After 160 hours at 20° no separation of the sugars in the racemic mixture was observed.

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