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Chromatographic Separation and Quantitative Determination of Monosaccharides

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The identification of the single monosaccharide components occurring in polysaccharides and their quantitative determination used in the past to be a quite difficult task. The introduction of partition chromatography for carbohydrate analysis completely changed the situation. The single monosaccharides can now easily be separated, and minute amounts of them determined quantitatively by accurate colorimetric methods.

Prior to the introduction of partition chromatography many attempts were made to separate different sugar derivatives by means of adsorption and elution (see Binkley and Wolfrom [1]). The procedures used, however, were far from satisfactory.

Partridge [2] in 1946 first used paper chromatography for the qualitative analysis of sugar mixtures and Flood, Hirst and Jones [3] in 1948 developed a paper chromatographic method for the quantitative determination of single monosaccharides. A number of drops of the solution to be analyzed were placed along the starting line on the filter paper, one of them some distance apart from the others. This last drop was used for the qualitative identification of the various components. These being identified, strips containing the various monosaccharides were cut from the dried filter paper parallel to the starting line. The strips were eluted and the amount of sugar determined quantitatively. The technique had its limitations as some pairs of sugars such as glucose and galactose, ribose and lyxose as well as ribose and fucose migrate with almost equal speeds, thus making separation difficult.

The first separation of unsubstituted monosaccharides on a column of powdered cellulose was performed by Hough, Jones and Wadman [4] in 1948. After further development their technique [5] permitted the isolation of sufficient amount of sugar for qualitative identification of the components as well.

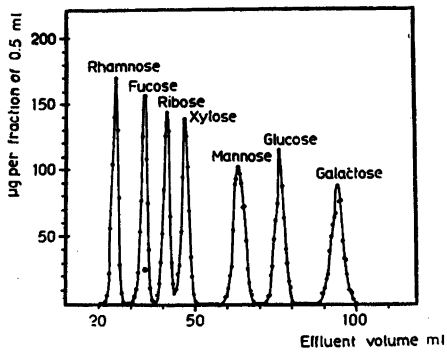

Fig. 1. Separation of seven monosaccharides on a 20 × 0.9 cm starch column. Solvent butanol : propanol : water 4 : 1 : 1.

Khym and Zill [6] separated several monosaccharides on columns of ion exchange resins using a borate buffer as eluent.

The present author has elaborated a technique for the separation and quantitative colorimetric estimation of the ordinary pentoses and aldohexoses. A starch column was used with a *n*-butanol : *n*-propanol : water mixture as the moving phase. Equal fractions of the effluent were collected and their content of sugar was determined colorimetrically directly on the solvent mixture as described by Gardell [7]. The separation could be visualized by constructing concentration-effluent curves (see Fig. 1). By summing the quantities found in the separate fractions the total amount of any individual sugar could be obtained, and the effluent volume for each peak in the curve indicated the nature of the individual sugar. If a column of a definite length is used the effluent volume for a monosaccharide is fairly constant. In Fig. 1 the separation of seven monosaccharides on a 20 × 0.9 cm starch column, and their quantitative analysis are demonstrated. (Run no. 2 in Table 1.)

Table 1. Recovery

Running no.	Rhamnose			Fucose			Ribose			Xylose			Mannose			Glucose			Galactose		
	Added µg	Found µg	Per cent recovery	Added µg	Found µg	Per cent recovery	Added µg	Found µg	Per cent recovery	Added µg	Found µg	Per cent recovery	Added µg	Found µg	Per cent recovery	Added µg	Found µg	Per cent recovery	Added µg	Found µg	Per cent recovery
1	338	358	105.9	1056	1084	102.7	175	170	97.1	861	925	107.4	372	355	95.4	1061	1111	104.7	368	360	97.8
2	720	746	103.6	734	690	94.0	745	698	93.7	726	708	97.5	802	834	104.0	702	703	100.1	746	746	100.0
3	674	728	108.0	104	97	93.3	336	347	103.3	384	390	101.6	890	848	95.3	365	359	98.4	346	324	93.6

EXPERIMENTAL

The *starch* and the *solvents* were purified routinely as described by Åqvist [8 p. 1035].

Preparation of the column: About 9 g of starch to be used in the 20 × 0.9 cm column was suspended in 25 ml of dry butanol to which had been added sufficient water to bring the total water present to 30 per cent of the weight of the anhydrous starch.

The suspension was filled into the glass tube, 50 × 0.9 cm, and left to settle under an air pressure of 5 cm Hg. When all the butanol had passed through the column, 200 ml of the butanol : propanol : water mixture (4 : 1 : 1) was run through under an air pressure of 10 cm Hg.

The hydrolysate of the polysaccharide was concentrated to a syrup *in vacuo* and excess hydrochloric acid was removed by repeated evaporation to dryness *in vacuo* after adding portions of water. Any insoluble residue was removed by centrifugation prior to the last evaporation to dryness.

Amino sugars and other ions occurring in quantities give rise to droplets of water in the solvent mixture to be applied and disturb the chromatographic separation. They were therefore removed by passing the hydrolysate before concentration to dryness through columns of cation and anion exchange resins.

The dry residue from the hydrolysate was dissolved in one volume of water (usually 1 ml), followed by one volume of dry propanol and four volumes of dry butanol. If the solution was not clear 0.4 ml of ethyl alcohol was added and the solution was made up to volume (10 ml) with the same solvent mixture. One ml of solution should contain 0.5—3 mg of each sugar component.

0.2—0.4 ml of the solution containing 100—1 200 μg of each component was run on to the top of the column and allowed to run down the column under an air pressure of 7—8 cm Hg, followed by 2 × 0.1 ml of the pure solvent mixture. Solvent mixture was then allowed to run through the column at a speed of 2 ml per hour, this requiring an air pressure of 10 cm Hg if the packing of the column was correct.

Collecting the effluent: Fractions of 0.5 ml of the solution leaving the column were collected. Since the colorimetric analysis to follow demands a definite composition of the solvent mixture the moving parts of the fraction collector were housed in a chamber saturated with the vapour of the solvent mixture (see Carlander and Gardell [9]). The fraction collector was started at the moment when the hydrolysate was applied to the column.

For quantitative determination of the amount of sugar present in the different fractions the method elaborated by Gardell [7] using the aniline-trichloracetate reagent was used. In this case, when 0.5 ml of effluent had been collected in each fraction, 0.5 ml of 96 per cent alcohol was added before adding the reagent. This was done with all the test solutions, standards, blanks and reference solutions.

Different sugars give different colour intensities with the reagent used. Therefore the nature of the sugar in each fraction was deduced from the effluent volume, which is fairly constant for columns of a definite length and width. The effluent volume was determined empirically with samples of pure monosaccharides. The columns to be compared were prepared with starch from the same batch.

All the readings of the tests as well as of the standards and of the blanks were made against a non-boiled reference solution consisting of 0.5 ml of the solvent mixture to which 0.5 ml of alcohol, 1 ml of the reagent and 2 ml of alcohol had been added. The optical density of the blank was subtracted from all the other values. The blank for the standards

had a composition similar to the reference solution but had been boiled, together with the standards.

The blank for the test solutions had been treated similarly but the 0.5 ml of the solvent mixture used had passed through the starch column, as described by Gardell [7, p. 1013].

DISCUSSION

When analyzing small amounts of sugars it is necessary to get sharp peaks in the effluent volume-concentration curves so as to be able to identify the single components. Preliminary experiments showed that this goal cannot be reached with the cellulose column which Hough, Jones and Wadman[4] used for separating larger amounts of the monosaccharides. Stein and Moore [10] showed very convincingly that starch columns give well reproducible chromatograms. We found the starch column still more advantageous, because, provided it is prepared exactly as described by Stein and Moore, it gives an improved separation of ribose from fucose, of xylose from ribose and of glucose from galactose. However different kinds of starch demand different amounts of water for saturation. When adequately packed, a starch column of 20 cm length allowed 1.5—2 ml of the butanol : propanol : water mixture to run through per hour under an air pressure of 10 cm Hg. The starch columns should be used only once. On repeated use the separation proved less satisfactory.

The composition of the solvent mixture, butanol : propanol : water (4 : 1 : 1) and the length of the column used allowed a good and quick separation of the monosaccharides occurring in the animal mucopolysaccharides. The water in the solvent mixture did not disturb the colorimetric analysis.

On such a column, 20 × 0.9 cm, the seven sugars rhamnose, fucose, ribose, xylose, mannose, glucose and galactose were completely separated. If arabinose was present, it preceded the mannose but was not completely separated from it. Lyxose came between ribose and xylose partly mixed with them. If only hexoses are present they can be separated in half the time on a shorter column of *e.g.* 10 cm length. If a component is not present in its expected place in the chromatogram, its presence can be excluded within the sensitivity limits of the colorimetric analysis. The identification of a component depends of course upon the accuracy with which its effluent volume can be determined. To be certain, a pure specimen of any component should be quantitatively recovered, if added to the hydrolysate, without any change in the symmetry of the concentration-effluent curve.

Ketoses cannot be analyzed with the aniline-trichloracetate method. On a column of this size 100—1 200 μg of each of the single monosaccharides

can be analyzed. For those components which are eluted most slowly the lower limit is higher due to the flatter shape of the curves at the end of the run. For the same reason a larger quantity of the hydrolysate than that given above cannot be applied as the components appearing first in the chromatogram would be found in concentrations so high as to be outside the range of the colorimetric method.

By using larger columns, larger fractions of the effluent can be collected, and this permits duplicate analyses on the single fractions and even isolation of material for chemical identification. For large scale preparative work cellulose columns are to be preferred for in spite of their poor resolving power they have a greater capacity and yield less impurities.

RESULTS

The yield in some experiments with pure specimens of monosaccharides are given in Table 1.

SUMMARY

A chromatographic technique has been elaborated for separating methylpentoses, pentoses and aldohexoses in amounts of 100 to 1 200 μg of each component as they occur in hydrolysates of polysaccharides and mucopolysaccharides. The separation was performed on a starch column using butanol: propanol : water (4 : 1 : 1) as the moving phase.

The effluent was collected in 0.5 ml portions with an automatic fraction collector, and the amount of sugar present in each fraction was determined quantitatively colorimetrically directly on the solvent mixture leaving the column.

Concentration-effluent curves were constructed allowing a qualitative identification of the components in the hydrolysate. By summing the amounts in each fraction containing a single sugar the amounts of the various components could be determined quantitatively.

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