A SOLVENT FOR QUALITATIVE AND QUANTITATIVE DETERMINATION OF SUGARS USING PAPER CHROMATOGRAPHY

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QUALITATIVE ANALYSIS

In carrying out studies of the carbohydrate fraction of wood[1], we have found it necessary to have a rapid and accurate method for the separation and qualitative and quantitative determination of the five principal sugars constituting this fraction: glucose, galactose, mannose, arabinose and xylose.

The classical methods of analysis, while adequate for the determination of a single sugar, are not suitable for the determination of a mixture. In fact, neither the technique of precipitation with specific reagents nor the method of selective sugar fermentation can be used to solve the problem of the separation of the five sugars.

PARTRIDGE'S method[2] of paper chromatography adapted from the method for the determination of amino acids[3], overcomes some of the difficulties, mainly by the simplicity of its technique. In fact many workers have used chromatographic analysis for the study of the hemicelluloses and of the composition of analytic α-cellulose. We can also say that, although this method is little used at present, it can give good results in the study of the composition of wood cooking liquors.

The method of paper chromatography did not previously completely solve the problem of the separation of sugar mixtures; in fact practically none of the eluent solvents used by other workers[4-7] gave a satisfactory result when used for a complete and rapid separation of the five sugars in the mixture. The solvents commonly used are based on butanol: the acid ones separate mannose from arabinose but not glucose from galactose; the basic ones separate glucose from galactose but not arabinose from mannose. For quantitative analysis it would be necessary to make two successive elutions using both acid and basic solvents.

To achieve a good separation, BOGGS[8] employed a double elution method. Such a determination, carried out at room temperature, requires about 40 hours, which we consider an excessive amount of time.

The purpose of this work was to find a solvent whose use would make possible the separation of monosaccharides from mixtures in reasonable time.

We began by examining numerous solvents suggested by various authors using the method of descending and ascending paper chromatography. For all the solvents we examined, we found that the method of descending chromatography produced

better separation than the ascending method, although the spots were somewhat elongated.

Furthermore, for all the solvents we found that better separation of monosaccharides is obtained using the system of multiple elutions: this consists of repeatedly drying and eluting the paper with fresh eluent.

The advantages obtained by using this method, which requires constant attention, are not such as to make it preferable to the normal elution process even though the time consumed is almost the same; this became more obvious when the ascending method was used.

In fact, in this case an increase of elution time over that required for the eluent to reach the top of the sheet is useless as any increase in time results in the production of diffused spots with lateral elongation.

A series of acid and basic eluents based on butanol, were tried using different proportions of: butanol–acetic acid–water, and butanol–pyridine–water.

The migration velocities of the monosaccharides were increased by substituting ethyl alcohol for the acid or the base, but the separation was still unsatisfactory.

This type of solvent gave good results when used for the separation of oligosaccharides, but not with monosaccharides, where the velocity of migration is too great.

We examined the possibilities of low viscosity solvents based on ethyl acetate, while realizing that double phase solvents give better results than monophase solvents.

The solvent mixture consisting of ethyl acetate, acetic acid and water gave fairly good results for the separation of mannose and arabinose; the separation of glucose and galactose was far better than with other solvents, but still unsatisfactory.

A basic mixture, ethyl acetate, pyridine and water gave the best results.

Using this mixture in the proportions: 3.6:1:1.15 and the descending method we obtained a complete separation of five principal sugars with elution times varying from 18 to 25 h at room temperature (about 20°). Fig. 1 shows a chromatogram of a mixture of six sugars obtained in 18 h elution at a laboratory temperature of 20°. Very good reproducibility of results was obtained without taking any special precaution to maintain constant conditions.

At 30°, a very good chromatogram was obtained in 15 h: naturally at this temperature we have to use a thermostatically controlled oven, but the spots are of good shape: round and compact, without tails after development.

Diffused spots and tailing can also be partially avoided by cutting the paper exactly perpendicular to the main fiber direction and then carrying out the chromatography at room temperature, as it was done for the chromatogram in Fig. 1.

Good results as regards roundness and compactness of spots can also be obtained by cutting the paper obliquely to the main fiber direction.

The presence of mineral salts (Na_2SO_4) does not influence the distinct separation of various monosaccharides with the solvent used, although the spots may be somewhat less regular and compact.

The best separations were obtained using drops of 2 μl of sugar solution at a concentration of each monosaccharide varying from 10 μg/μl to 2 μg/μl.

After elution and drying we used the modified PARTRIDGE's developing agent consisting of acid aniline phthalate in water-saturated butanol. The solution was

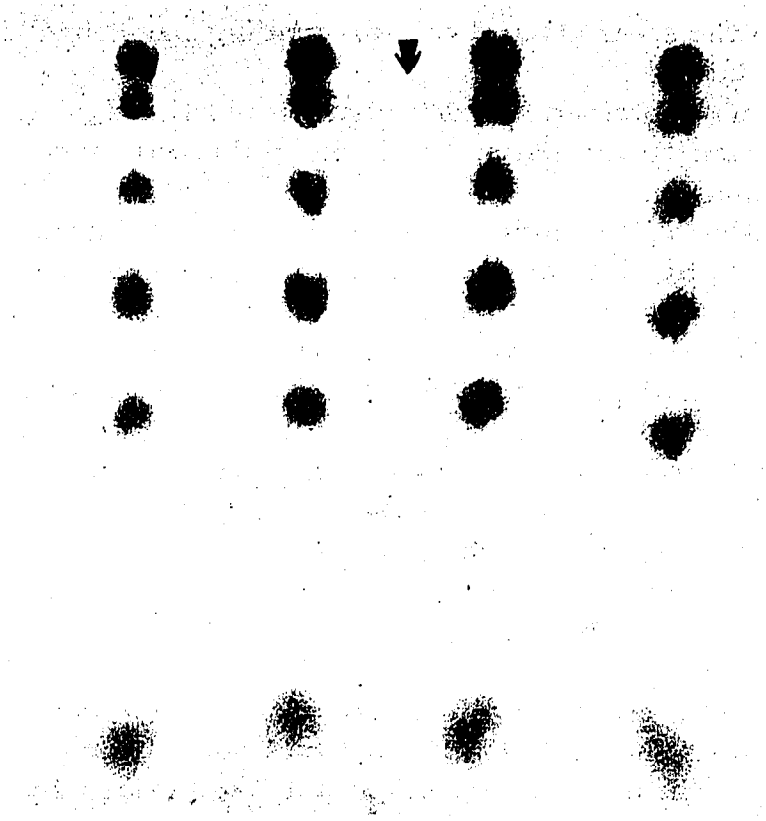

Fig. 1. Chromatogram of a mixture of six sugars. Elution time at 20° = 18 h.

freshly prepared before each determination, thus preventing any discoloration of the solution and obtaining maximum contrast between the spots and the paper.

QUANTITATIVE ANALYSIS

After overcoming, by the use of our solvent, the problem of the distinct and rapid separation of the monosaccharides, we turned our attention to the possibilities of quantitative determinations.

Using a micropipette, we placed a 2 μl drop of the unknown sugar solution at two places on the departure line of the chromatography sheet; at five other places we placed 2 μl drops of solution of known sugar content. The process of chromatography was carried out as described in the experimental section.

The five drops of known solutions, containing the monosaccharides in various concentrations, were used to obtain a rough approximation of the concentration of the sugars in an unknown solution. After this previous observation, calibration solutions were prepared for the quantitative analysis.

For every single sugar five spots were obtained, whose colour intensity was directly proportional to the sugar concentration.

We preferred the method of direct measurement of the total colour density of the spots.

After development and drying (oven at 105°), intensity measurements were carried out on both the spots (I) and the surrounding paper background (I_0) and the ratio I_0/I was calculated.

A straight line was obtained for each sugar on plotting I_0/I against concentration (μg/2 μl) on semi-logarithmic paper; and the unknown sugar concentration was obtained by interpolation.

As the relationship I_0/I is unfluenced by the physical characteristics of the paper used for chromatography (transparency, texture) and colour after the development, it was necessary to construct a separate graph for each sheet. Generally, the spots photographed by transparency, are less compact than those observed by direct observation or by normal photography. This may be a possible cause of errors in quantitative analysis made by the direct measurement of the total colour density.

This is a rapid method which does not require the use of an apparatus more complicated than a normal photoelectric densitometer.

The main problem we have met using this meter is the difficulty in obtaining chromatograms with regular circular spots of sufficiently small dimensions. In fact the search unit has an aperture of fixed diameter (2 cm) and the spots must not have a greater diameter than this.

This difficulty was overcome by the use of our solvent which gave good resolution of the spots. The results shown in Table I, were obtained using this solvent and thus demonstrate its properties. Better results could be obtained by the use of a more accurate light meter.

It was also necessary to spray the developer as uniformly as possible and carry out the colour density measurements as soon as possible after development. In fact the sheets, after development and drying, have a tendency to become yellow on exposure to the air.

In order to establish the precision of the method, 12 chromatograms were prepared with a descending method, using the following solution of known composition (A):

Glucose	200 mg/100 ml
Galactose	250 mg/100 ml
Mannose	250 mg/100 ml
Arabinose	250 mg/100 ml
Xylose	750 mg/100 ml

Each chromatogram was prepared by placing on the departure line, as mentioned before, 2 drops each of 2 μl of solution A and 5 drops of the standard calibration solutions.

The values for each single sugar obtained from the two drops were averaged. The sugar content given by each spot of solution A varied from 4 μg of glucose to 15 μg of xylose. The results obtained are given in Table I.

TABLE I

PRECISION OF THE METHOD. RESULTS FROM 12 ANALYSES

Test	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*Average value*	*Standard deviation*	*% Deviation from theory*	*Coefficient of variation*
Glucose	195	195	200	190	195	205	205	205	205	200	200	210	200.41	± 5.79	+ 0.205	± 2.89
Galactose	255	240	240	250	237	265	245	240	260	250	250	250	248.50	± 8.60	— 0.600	± 3.46
Mannose	245	255	250	250	240	250	250	235	250	250	235	270	248.33	± 9.34	— 0.670	± 3.76
Arabinose	256	245	250	248	248	245	250	240	240	250	240	250	246.90	± 5.01	— 0.840	± 2.03
Xylose	738	760	760	725	750	750	730	730	760	737	740	750	744.16	± 12.57	— 0.780	± 1.69

Calculation of coefficient of variation:

$$S^2 = \frac{\Sigma (x - \bar{x})^2}{N - 1}.$$

$\Sigma (x - \bar{x})^2$ = sum of squared deviation of sample mean.

$\bar{x}$ = mean.

$\frac{100 \cdot S}{\bar{x}}$ = coefficient of variation.

N = number of tests.

EXPERIMENTAL DETAILS

Standard laboratory apparatus was used throughout this work. Chromatography was carried out in a glass air-tight vessel of dimensions 20 × 20 × 60 cm^3.

The troughs holding the eluent were also of glass and supported by steel uprights.

The chromatography paper used was Whatman No. 1 grade; and in each case the sheets were cut to the dimensions 22 × 46 cm^2.

The Photovolt densitometer Model 610 with a Blue Filter Wratten 49 was used to measure the colour density of the spots.

A descending method of chromatography was employed.

The drops of the sugar solutions were placed with a 2 μl micropipette along a pencil line, drawn across the width of the sheet at 10 cm from one end.

The sheets were introduced into the chromatography vessel and weighted by fastening to the ends of the sheets glass weights which served to increase the rate of flow of the solvent through the paper.

The solvent was freshly prepared by shaking, in a separating funnel, ethyl acetate, pyridine, water in the proportion 3.6:1:1.15. The mixture was allowed to separate into two layers: the lower layer was placed in the bottom of the chromatography tank to saturate the atmosphere. The upper layer was placed in the elution troughs.

The sheets after elution, which could take from 18 h (qualitative analysis) to 25 h (quantitative analysis) at a temperature of about 20°, were taken from the tank and dried at room temperature.

The sheets were developed by spraying, as uniformly as possible with a fine spray of developing solution under pressure from a nitrogen cylinder.

The developer was freshly prepared by dissolving 2.5 g of acid aniline phthalate in 100 ml of water-saturated butanol.

The acid aniline phthalate was prepared by dissolving phthalic acid in warm alcohol and then adding aniline. The solution was cooled and the precipitated acid aniline phthalate was recrystallized from alcohol and washed with ether.

By preparing the developer in this way the inconvenience of discoloration due to the presence of aniline impurities, which cannot be completely removed by distillation, was overcome.

The chromatography sheets, after spraying and development, were dried in an oven, in which the air was circulated, for a period of 5 min at 105° ± 1°.

Measurement of spot colour density, using a Photovolt densitometer, was carried out immediately after the spraying.

For a development time of 22 h (at a room temperature of about 20°) the movement of single sugars relative to the movement of glucose (R_{Glu}) was measured; the results are given below:

Glucose	= 1.00
Galactose	= 0.80
Mannose	= 1.30
Arabinose	= 1.64
Xylose	= 2.18
Rhamnose	= 4.30

The reproducibility of these results was found to be good and independent of room conditions.

Comparing these values with those given in the literature for other solvents a definite improvement in the separation was noted.

For 18 h elution time (always at room temperature) the R_{Glu} values were somewhat modified:

Glucose	= 1.00
Galactose	= 0.80
Mannose	= 1.30
Arabinose	= 1.62
Xylose	= 2.14

Experiments were also carried out at 30°, by placing the chromatography vessel in a thermostatically controlled oven.

In this case the initial spot of 2 μl contained 360 μg of xylose, 15 μg of galactose, 28 μg of glucose, 22 μg of mannose, 10 μg of arabinose and 7.5 μg of rhamnose.

The R_{Glu} values were calculated for 15 h elution time:

Glucose	= 1.00
Galactose	= 0.79
Mannose	= 1.30
Arabinose	= 1.57
Xylose	= 2.01
Rhamnose	= 3.16

The initial spots of sugars solutions of 2 μl and which contained every sugar in the mixture, in quantities varying from 40 μg to 8 μg, gave the best separations.

The rhamnose, traces of which were definitely found in the hydrolysate of poplar sawdust, has been introduced in some calibration tests to study the possibility of its quantitative determination, in the presence of large quantities of other sugars.

SUMMARY

The object of this work was to find a rapid and precise method for the separation of the principal monosaccharide constituents of the carbohydrate fraction of wood: glucose, galactose, mannose, arabinose and xylose.

Discarding the imprecise methods of classical analysis, we have considered the possibility of the application of paper chromatographic methods.

None of the eluents suggested in the literature gives a satisfactory solution to this problem.

We have studied the use of a solvent composed of a mixture of ethyl acetate, pyridine and water in the proportions 3.6:1:1.15; we have found that this eluent gives a complete separation of the sugars and allows both qualitative and quantitative determinations.

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