Colorimetric Method for Determination of Sugars and Related Substances

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Simple sugars, oligosaccharides, polysaccharides, and their derivatives, including the methyl ethers with free or potentially free reducing groups, give an orange-yellow color when treated with phenol and concentrated sulfuric acid. The reaction is sensitive and the color is stable. By use of this phenol–sulfuric acid reaction, a method has been developed to determine submicro amounts of sugars and related substances. In conjunction with paper partition chromatography the method is useful for the determination of the composition of polysaccharides and their methyl derivatives.

COLORIMETRIC tests for reducing sugars and polysaccharides have been known for a considerable time. The reagents such as 1-naphthol (*33*) for carbohydrates in general; benzidine for pentoses and uronic acids (*27, 49, 50*); naphthoresorcinol for uronic acids (*51*); and resorcinol (*43*), naphthoresorcinol (*39*), and resorcinol disulfonic acid (*31*) for ketoses are well-known examples of colorimetric tests that may be carried out in acid solution. Such tests as these and modifications of them using aromatic amines and phenols (*4, 22, 38*) have recently gained added importance since the extensive development of partition chromatography for the separation and characterization of minute amounts of sugars and their derivatives (*1, 4, 8, 11, 12, 17, 18, 21–23, 26, 36, 39, 47*). Polyols and carbohydrates with a reducing group may be detected by the Tollens silver reagent (*39, 52*), perhaps one of the best reagents in the art of chromatography. Reducing sugars are also detectable by picric acid (*7, 17*), 3,4-dinitrobenzoic acid (*5*), 3,5-dinitrosalicylic acid (*6, 32, 48*), *o*-dinitrobenzene (*17, 40*), and methylene blue (*54*), while diazouracil is said to be specific for sucrose as well as oligosaccharides and polysaccharides containing the sucrose residue (*42*).

Volumetric procedures involving the use of potassium ferricyanide (*19*), ceric sulfate (*45*), copper sulfate (*16, 44*), and sodium hypoiodite (*20*) are applicable to the determination of small amounts of reducing sugars after separation by partition chromatography. However, experience shows that these methods require considerable skill and are time-consuming and sensitive to slight variation in the conditions.

The anthrone (*13, 14, 28, 34, 35, 53*) and the 1-naphtholsulfonate (*10*) reagents are excellent for standard sugar solutions (*34*), but, when applied to the analysis of sugars separated by partition chromatography, the presence of only traces of residual solvent developer may render them useless. Most sugars can be separated on filter paper by a phenol-water solvent (*39*), but they cannot then be determined by the anthrone reagent because residual phenol, held tenaciously in the paper, interferes with the green color produced by the anthrone reagent. Moreover, the anthrone reagent is expensive and solutions of it in sulfuric acid are not stable (*30, 34*). The anthrone method also suffers from the disadvantage that, while it is satisfactory for free sugars and their glycosides, it is of limited use for methylated sugars and the pentoses. Although butanol–propionic acid–water is an excellent solvent for separating the disaccharides (*4*), the residual propionic acid interferes with the 1-naphtholsulfonate method. Aniline phthalate (*38*) and aniline trichloroacetate (*17*) have been utilized for the colorimetric determination of sugars and their derivatives (*2, 3*); these reagents, however, are unsatisfactory for ketoses.

Phenol in the presence of sulfuric acid can be used for the quantitative colorimetric microdetermination of sugars and their methyl derivatives, oligosaccharides, and polysaccharides (*15*). This method is particularly useful for the determination of small quantities of sugars separated by paper partition chromatography with the phenol-water solvent and also for those sugars separated with solvents which are volatile—e.g., butanol-ethanol-water (*39*), ethyl acetate–acetic acid–water (*26*), or methyl ethyl ketone–water (*4, 39*). The method is simple, rapid, and sensitive, and gives reproducible results. The reagent is inexpensive and stable, and a given solution requires only one standard curve for each sugar. The color produced is permanent and it is unnecessary to pay special attention to the control of the conditions.

DETERMINATION OF CONCENTRATION OF PURE SUGAR SOLUTIONS

Reagents and Apparatus. Sulfuric acid, reagent grade 95.5%, conforming to ACS specifications, specific gravity 1.84.

Phenol, 80% by weight, prepared by adding 20 grams of glass-distilled water to 80 grams of redistilled reagent grade phenol. This mixture forms a water-white liquid that is readily pipetted. Certain preparations have been known to remain water-white after a years' storage, while others turn a pale yellow in 3 or 4 months. The pale yellow color that sometimes develops does not interfere in the determination, inasmuch as a blank is included.

Coleman Junior, Evelyn, Klett-Summerson, or Beckman Model DU spectrophotometers. All were used with satisfactory results in this investigation.

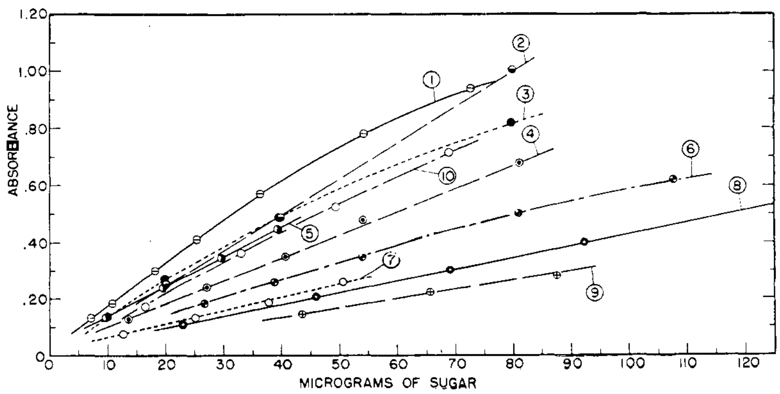

Figure 1. Standard curves

1. D-Xylose, Coleman Jr., 480 mμ, 17 mg. of phenol
2. D-Mannose, Beckman Model DU, 490 mμ, 40 mg. of phenol
3. D-Mannose, Evelyn, filter No. 490, 40 mg. of phenol
4. D-Galactose, Coleman Jr., 490 mμ, 33 mg. of phenol
5. L-Arabinose, Coleman Jr., 480 mμ, 17 mg. of phenol
6. D-Galacturonic acid, Coleman Jr., 485 mμ, 17 mg. of phenol
7. L-Fucose, Coleman Jr., 480 mμ, 40 mg. of phenol
8. D-Glucurone, Coleman Jr., 485 mμ, 17 mg. of phenol
9. 2,3,4,6-Tetra-*o*-methyl-D-glucose, Coleman Jr., 485 mμ, 17 mg. of phenol
10. D-Glucose, Beckman Model DU, 490 mμ, 100 mg. of phenol

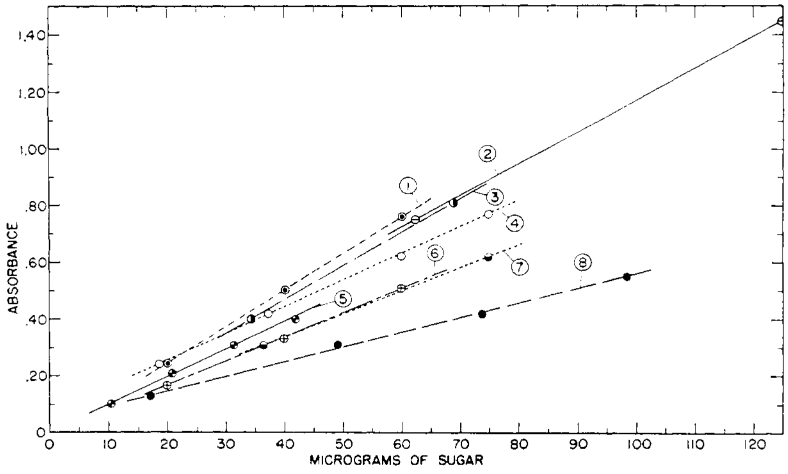

Figure 2. Standard curves

1. Sucrose, Beckman Model DU, 490 mμ, 100 mg. of phenol
2. Potato starch, Beckman Model DU, 490 mμ, 100 mg. of phenol
3. Dextran from *Leuconostoc mesenteroides* strain NRRL 512, Beckman Model DU, 490 mμ, 103 mg. of phenol
4. D-Glucose, Evelyn, filter No. 490, 80 mg. of phenol
5. L-Rhamnose, Coleman Jr., 480 mμ, 40 mg. of phenol
6. Raffinose, Beckman Model DU, 490 mμ, 100 mg. of phenol
7. D-Fructose, Beckman Model DU, 490 mμ, 200 mg. of phenol
8. 2-Deoxy-D-ribose, Coleman Jr., 490 mμ, 80 mg. of phenol

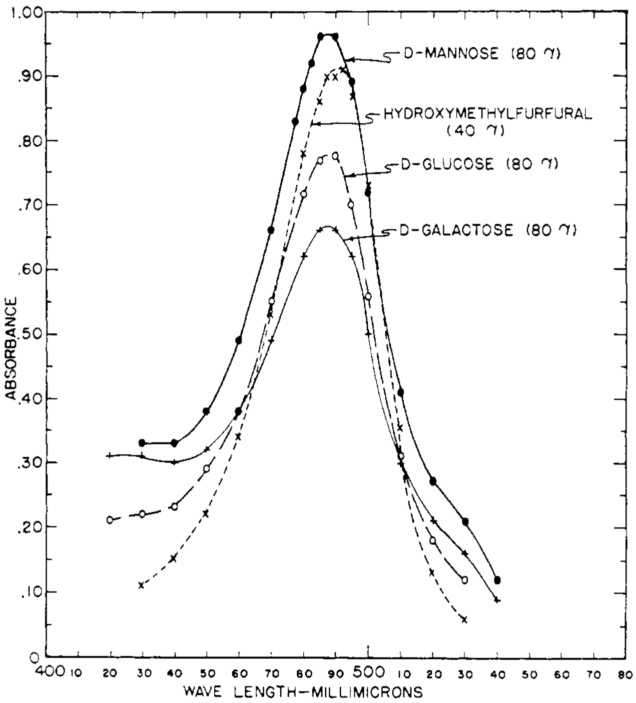

Figure 3. Absorption curves

Fast-delivery 5-ml. pipet, to deliver 5 ml. of concentrated sulfuric acid in 10 to 20 seconds. This is easily prepared by cutting a portion of the tip of a standard 5-ml. pipet.

Series of matched colorimetric tubes, internal diameter between 16 and 20 mm. This diameter will allow good mixing without dissipating the heat too rapidly. A high maximum temperature is desired because it increases the sensitivity of the reagent.

Series of micropipets delivering 0.02, 0.05, and 0.1 ml. The type described by Pregl (*41*) is satisfactory.

Procedure. Two milliliters of sugar solution containing between 10 and 70 γ of sugar is pipetted into a colorimetric tube, and 0.05 ml. of 80% phenol (adjust amount according to Figures 9 and 10) is added. Then 5 ml. of concentrated sulfuric acid is added rapidly, the stream of acid being directed against the liquid surface rather than against the side of the test tube in order to obtain good mixing. The tubes are allowed to stand 10 minutes, then they are shaken and placed for 10 to 20 minutes in a water bath at 25° to 30° C. before readings are taken. The color is stable for several hours and readings may be made later if necessary. The absorbance of the characteristic yellow-orange color is measured at 490 mμ for hexoses and 480 mμ for pentoses and uronic acids. Blanks are prepared by substituting distilled water for the sugar solution. The amount of sugar may then be determined by reference to a standard curve previously constructed for the particular sugar under examination.

All solutions are prepared in triplicate to minimize errors resulting from accidental contamination with cellulose lint.

If it is desired to avoid the use of micropipets, the phenol may be added as a 5% solution in water. The amounts of reactants are then: 1 or 2 ml. of sugar solution, 1 ml. of 5% phenol in water, and 5 ml. of concentrated sulfuric acid. All other steps are the same as above.

Standard Curves. A series of typical standard curves is shown in Figures 1 and 2. Included in these figures are examples of some of the sugars usually encountered in carbohydrate studies—namely, pentose, deoxypentose, methylpentose, aldohexose, ketohexose, hexuronic acid, disaccharide, trisaccharide, and certain methylated derivatives. In order to test the method, the experiments were repeated on different days and by different operators. In all cases the variations between experiments and between operators were no more than 0.01 to 0.02 unit in absorbance, which was the same order of magnitude as the variation between the triplicate samples.

The experimental data for the various carbohydrates, except 2-deoxyribose, given in Figures 1 and 2 may be tabulated by calculating the value of a_s, the absorbance index, in the equation $A_s = a_s bc$ (Table I). The absorbance, A_s, is a dimensionless ratio equal to $\log_{10} \frac{T_{\text{solvent}}}{T_{\text{solution}}}$, where T is per cent transmittance, b is the length of light path, expressed in centimeters, and c is the concentration, in micrograms of sugar per milliliter of final volume.

Discussion of Results. Absorption Curves. The curves obtained by plotting absorbance *vs.* wave length (Beckman Model

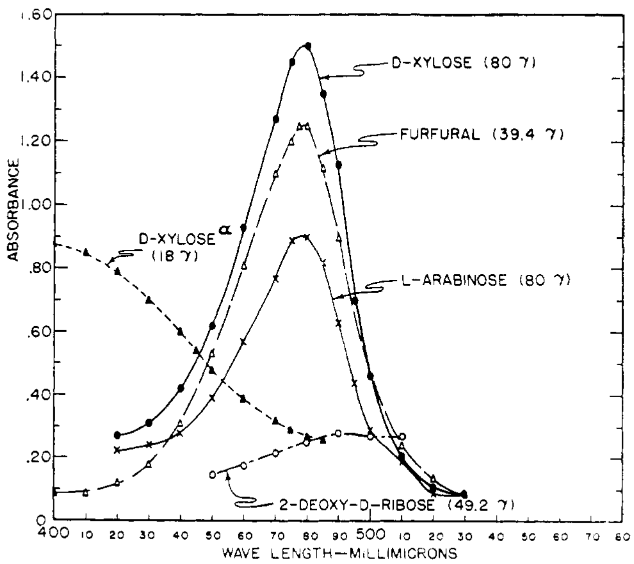

Figure 4. Absorption curves

A 0.1 ml. of butanol-ethanol-water chromatographic developing solvent (4 to 1 to 5, upper layer) was added in addition to the phenol

DU) are shown in Figures 3 to 8; the absorption curve is characteristic for each of the sugars described (*9, 25*). The pentoses, methylpentoses, and uronic acids have an absorption maximum at 480 mμ, while hexoses and their methylated derivatives have an absorption maximum at 485 to 490 mμ. Certain of the methylated pentose sugars and their methyl glycosides show selective absorption at about 415 to 420 mμ (Figure 8) and for this reason the colorimetric determination of 2,3,5-tri-*o*-methyl-L-arabinose and its methyl glycoside is best carried out at 415 mμ.

The D-xylose and furfural curves are very similar. Assuming that the amount of color is proportional to the amount of furfural present or produced, the conversion of D-xylose to furfural under the conditions of the test is 93% of theory.

Calculation of conversion of D-xylose to furfural

	M.W.	Micrograms	Absorbance
Furfural	96	39.46	1.25
D-Xylose	150	80	1.50

The percentage, P, of xylose converted to furfural in the reaction as measured by the intensity of color developed can be calculated as illustrated below:

$$P = \frac{1.50}{1.25} \times \frac{39.46}{96} \times \frac{150}{80} \times 100 = 92.5\%$$

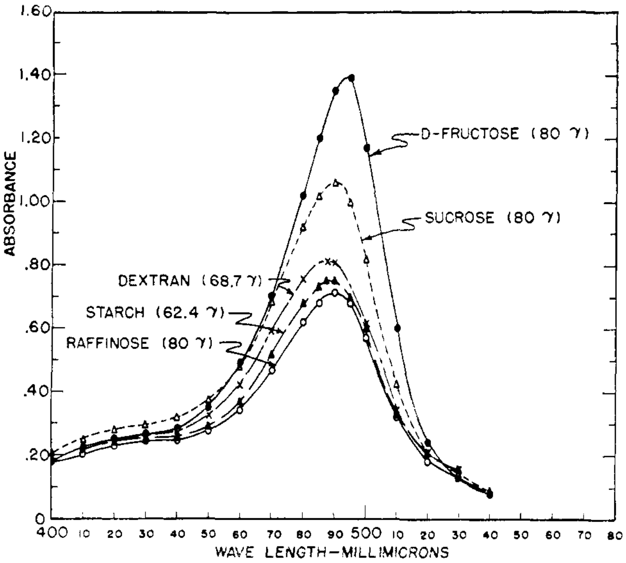

Figure 5. Absorption curves

Table I. Absorption Data for Certain Carbohydrates Determined by Phenol–Sulfuric Acid Reagent

Compound	Wt., γ	Phenol, Mg.[c]	Vol., Ml.	Light Path, Cm.	Instrument[a]	Wave Length, Mμ	Absorbance	a_s	Klett Reading[b]
D-Fructose	37.4	40	6.60	1	B	490	0.31	0.0547	
	42.4	51.6	6.61	1	B	490	0.35	0.0545	
	42.4	103	6.64	1	B	490	0.48	0.0752	
	42.4	154	6.68	1	B	490	0.52	0.0819	
	42.4	206	6.72	1	B	490	0.47	0.0902	
	42.4	310	6.80	1	B	490	0.58	0.0928	
D-Glucose	42.2	51.6	6.61	1	B	485	0.45	0.0704	
	42.2	103	6.64	1	B	485	0.448	0.0702	
	42.2	154	6.68	1	B	485	0.40	0.0632	
	80	40	6.60	1	B	487	0.78	0.0640	
Sucrose	53.6	40	6.60	1	B	490	0.48	0.0591	
	26.3	40	6.60	1	B	490	0.237	0.0594	
	35	100	6.64	1.6	C	490	0.395	0.0468	
5-Hydroxymethyl-2-furaldehyde	40	154	6.68	1	B	490	0.86	0.143	
	40	206	6.72	1	B	490	0.95	0.159	
	40	257	6.76	1	B	490	0.98	0.166	
Starch	62.4	103	6.64	1.27	K	Blue, No. 42	0.0328	0.00275	16.4
	124.8	103	6.64	1.27	K	Blue, No. 42	0.064	0.00268	32
	187.2	103	6.64	1.27	K	Blue, No. 42	0.096	0.00268	48
	312.0	103	6.64	1.27	K	Blue, No. 42	0.146	0.00236	73
	62.4	103	6.64	1	B	488	0.75	0.0799	
	124.8	103	6.64	1	B	488	1.45	0.0772	
Dextran	34.36	103	6.64	1.27	K	Blue, No. 42	0.0166	0.00252	8.3
	68.72	103	6.64	1.27	K	Blue, No. 42	0.0338	0.00257	16.9
	137.44	103	6.64	1.27	K	Blue, No. 42	0.0646	0.00246	32.3
	286.4	103	6.64	1.27	K	Blue, No. 42	0.1380	0.00252	69
	34.36	103	6.64	1	B	488	0.40	0.0774	
	68.72	103	6.64	1	B	488	0.81	0.0784	
D-Galacturonic acid	80	16	6.58	1.00	B	480	0.532	0.0439	
D-Mannurone	80	40	6.60	1.00	B	485	0.39	0.0322	
D-Glucurone	80	40	6.60	1.00	B	480	0.287	0.0237	
D-Galactose	80.2	40	6.60	1.00	B	487	0.664	0.0546	
D-Mannose	80	40	6.60	1.00	B	487	1.01	0.0835	
L-Arabinose	80	40	6.60	1.00	B	480	0.90	0.0742	
D-Xylose	80	40	6.60	1.00	B	480	1.50	0.1239	
L-Rhamnose	80	16	6.58	1.00	B	480	0.82	0.0674	
L-Fucose	80	16	6.58	1.00	B	480	0.35	0.0288	
Maltose	40	100	6.63	1.6	C	490	0.47	0.0492	
Raffinose	50	100	6.63	1.6	C	490	0.46	0.0381	
Lactose	50	100	6.63	1.6	C	490	0.355	0.0294	
2-*o*-Methyl-D-xylose	50	20	7.45	1.6	C	485	0.31	0.0289	
2,3-Di-*o*-methyl-D-xylose	58.5	20	7.45	1.6	C	480	0.39	0.031	
Methyl 2,3-di-*o*-methyl-D-xyloside	47.7	35	7.45	1.00	B	480	0.23	0.036	
Methyl 2,3-di-*o*-methyl-D-xyloside	47.7	35	7.45	1.00	B	415	0.21	0.0328	
2,3,5-Tri-*o*-methyl-L-arabinose	40	50	7.45	1.6	C	415	0.27	0.0314	
Methyl 2,3,5-tri-*o*-methyl-L-arabinoside	50	50	7.45	1.6	C	415	0.325	0.0302	
2,3-Di-*o*-methyl-D-glucose	80	40	6.60	1.00	B	485	0.76	0.0708	
2,3,6-Tri-*o*-methyl-D-glucose	53	40	6.60	1.00	B	485	0.555	0.0690	
2,3,4,6-Tetra-*o*-methyl-D-glucose	80	120	6.65	1.00	B	485	0.57	0.0474	
2,3-Di-*o*-methyl-D-mannose	50	50	6.57	1.6	C	485	0.39	0.0320	
2,3,6-Tri-*o*-methyl-D-mannose	50	50	6.57	1.6	C	485	0.37	0.0304	
2,3,4,6-Tetra-*o*-methyl-D-galactose	50	50	6.57	1.6	C	485	0.37	0.0304	

[a] B, Beckman Model DU; C, Coleman Junior; K, Klett-Summerson.

[b] Klett reading $= \frac{1000 \times \text{absorbance}}{2}$

[c] Actual weight of phenol. To find weight of 80% solution, divide by 0.8.

Calculation of final volume

2 ml. water	2
5 ml. sulfuric acid × 1.84	9.20
Total wt.	11.20 grams

Concn. of sulfuric acid after mixing $\frac{9.20 \times 0.95}{11.20} = 78\%$

Density of 78% sulfuric acid (20° C.) 1.7043

Volume of mixture $\frac{11.20}{1.70} = 6.57$ ml.

The addition of small amounts of phenol was considered to have a negligible effect on the density of the solution; hence, 0.1 ml. of 80% phenol would increase the volume by 0.06 ml.

2 ml. water	2
1 ml. 5% phenol in water	1
5 ml. sulfuric acid	9.2
Total wt.	12.2 grams

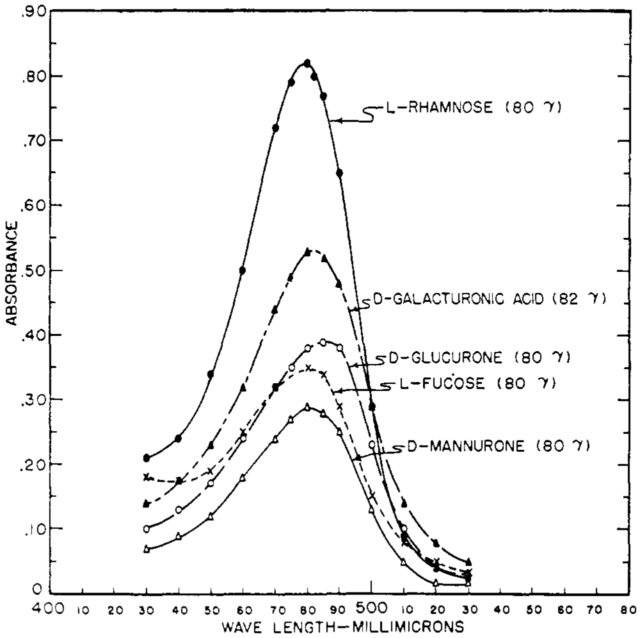

Figure 6. Absorption curves

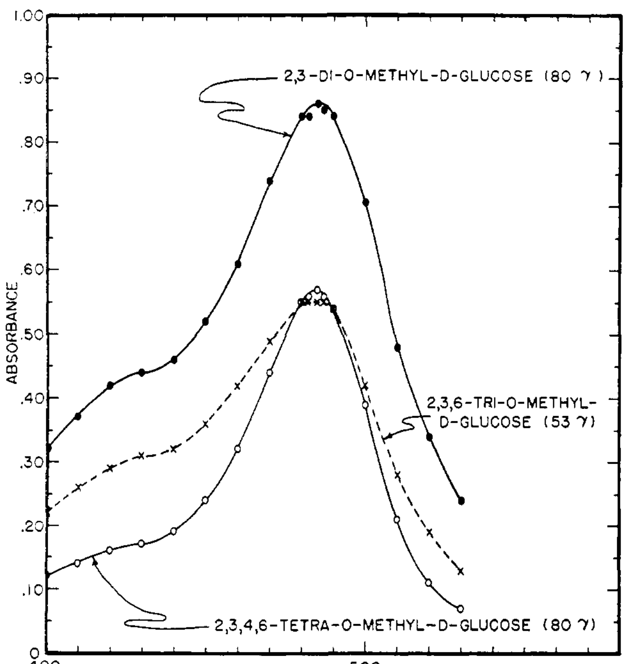

Figure 7. Absorption curves

Table II. Relationship between Index of Absorbance and Sugar Concentration as Determined by Different Instruments

Instrument	Approx. Band Width, Mμ	D-Mannose, γ	Light Path, Cm.	Absorbance	a_s
Beckman Model DU	0.5	80	1.00	1.01	0.0835
	0.5	40	1.00	0.495	0.0815
	0.5	20	1.00	0.25	0.0826
Coleman Jr.	50	41.1	1.6	0.45	0.0451
	50	20.5	1.6	0.24	0.0481
	50	10.2	1.6	0.11	0.0442
Evelyn	65	40	1.9	0.49	0.0426
	65	20	1.9	0.27	0.0464
	65	10	1.9	0.13	0.0473

Concn. of sulfuric acid $\frac{9.20 \times 0.95}{12.2} = 71.6\%$

Density at 20° C. 1.628

Volume of mixture $\frac{12.20}{1.628} = 7.48$ ml.

Effect of Variable Amounts of Phenol. The intensity of the color is a function of the amount of phenol added. As the amount of phenol is increased, the absorbance increases to a maximum and then usually falls off (Figures 9 and 10). When a paper chromatographic separation has been effected using phenol as a solvent, it will be found impractical to remove all of the phenol developer by air drying. This is not essential, though, because the curve of absorbance *vs.* amount of phenol is relatively flat after the maximum color intensity has been reached. Reproducible results can be obtained by operating at either side of the peak or at the peak as long as the amount of phenol added is controlled. This could conceivably form the basis for the analysis of mixtures of sugars—for instance, of D-mannose and D-glucose—by making two series of experiments, one at low and one at high phenol concentrations. The difference in readings is not large enough by itself except for rather crude estimations, but in combination with the variation in wave length of absorption maxima peaks between pentoses or uronic acids and hexoses, a satisfactory analysis might be devised.

A procedure using a somewhat similar idea, the rate of color development between sugars and the anthrone reagent, has been reported by Koehler (*28*).

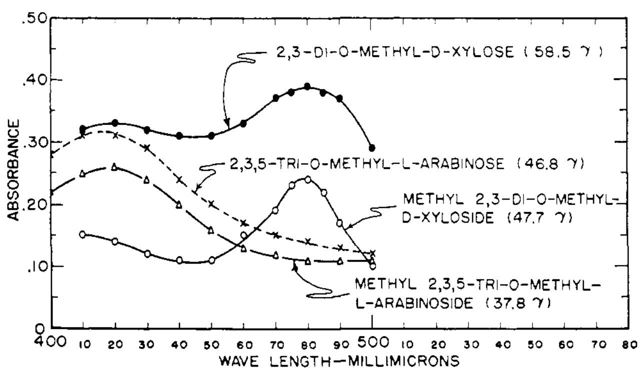

Figure 8. Absorption curves

Effect of Band Width. The absorbance, as is generally true in colorimetric determinations, is a function of the length of light path as well as the band width of the light source. As the band width becomes narrower, the observed absorbance becomes greater. If the values of the constant a_s are calculated from the equation $A_s = a_s bc$, the effect of the band width becomes apparent (Table II).

The higher the value of a_s, the more sensitive is the instrument. On this basis, the Beckman was the most sensitive instrument used; the others, however, perform well enough for routine analysis.

In the case of the Evelyn and the Coleman colorimeters, the value of a_s is not constant. This means that the plot of concentration *vs.* absorbance is not linear at the higher concentrations; however, it is very nearly linear at lower concentrations. The linearity of the plot of absorbance *vs.* concentration is extended to higher regions of concentration by operating at narrower band widths. The points obtained in the nonlinear region with the colorimeters passing wider bands are, nevertheless, reproducible (Table II).

ACCURACY OF METHOD. Under the proper conditions, the method can be expected to be accurate to within ±2%. This figure was obtained by plotting the results obtained by use of the Beckman Model DU spectrophotometer and comparing the amount of sugar actually present with that indicated by the plot. As mentioned previously, the narrow band width of the Beckman spectrophotometer makes it possible to extend the linearity of the standard curve. The percentage error is shown in Table III.

Table III. Accuracy of Phenol-Sulfuric Acid Method for Sugar Determination

Compound	Taken, γ	Found, γ	Error, %	Absorbance
Mannose	80	81	1.3	1.01
	40	39	2.5	0.495
	20	20	0.0	0.25
Galactose	80.4	79.5	1.1	0.665
	40.2	39.5	1.7	0.325
	21.4	21.5	0.5	0.175

Conclusions. The phenol-sulfuric acid method can be used to give reliable estimations of the sugar content of pure solutions. The colors produced are unusually stable, and possess a definite absorption peak. The amount of color produced at a constant phenol concentration is proportional to the amount of sugar present. The standard curves obtained by plotting the sugar concentration *vs.* the absorbance can be readily reproduced and, because of this, only one standard curve need be prepared for a given sugar. Furthermore, the reagents are inexpensive, stable, and readily available.

QUANTITATIVE ANALYSIS OF SUGARS BY PAPER CHROMATOGRAPHY

The application of qualitative paper chromatography to the separation of sugar mixtures has been extended to the field of quantitative analysis. Any sugars that can be separated by the technique of paper chromatography can be determined quantitatively by the colorimetric technique just described after elution from the paper (*13, 15, 29*). The principle is simple, but certain factors complicate the analysis. Probably the most serious of these is that carbohydrate impurities are extracted from the paper along with the sugar to be analyzed. This source of error is reduced greatly by the simple expedient of running a blank. The size of the blank reading may be reduced to about one half by washing the papers with distilled water containing about 1% ammonia (*37*). Another complicating factor is the introduction of cellulose lint during the elution procedure, but this can be eliminated entirely by careful filtration.

A procedure similar to the one described herein is reported by Dimler and others (*13*). However, their elution procedure is considerably more complicated than the one used in this work. Furthermore, the best colorimetric technique at the disposal of these workers was the anthrone method, the disadvantages of which have already been explained.

Washing of Paper. The following experiment illustrates how the soluble carbohydrate fraction present in filter paper may be reduced by washing. This fraction cannot be entirely washed out (*24*), and seems to increase after the washed paper is allowed to dry (*46*). Other work (*24*) in this laboratory has shown that the soluble carbohydrate fraction of filter paper is of the nature of a pentosan. The further study of this carbohydrate material will form the subject of another communication.

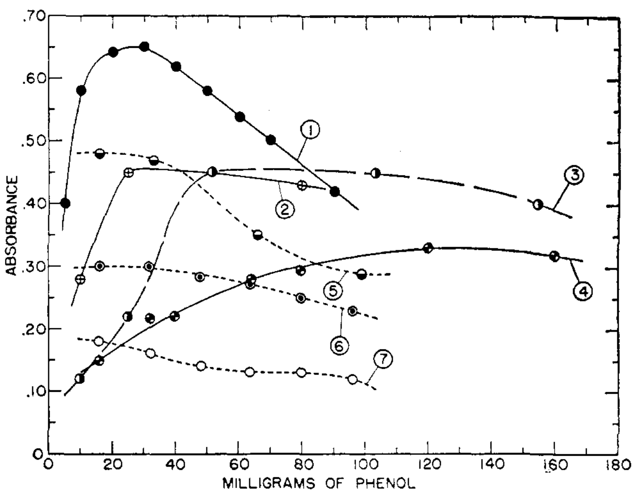

Figure 9. Absorbance *vs.* amount of phenol

1. D-Xylose, 40 γ, Coleman Jr., 480 mμ
2. D-Mannose, 41 γ, Evelyn, filter No. 490
3. D-Glucose, 42 γ, Beckman Model DU, 485 mμ
4. 2-Deoxy-D-ribose, 49 γ, Coleman Jr., 490 mμ
5. D-Galactose, 54 γ, Coleman Jr., 490 mμ
6. L-Rhamnose, 52 γ, Evelyn, filter No. 490
7. L-Fucose, 25 γ, Coleman Jr., 480 mμ

A piece of Whatman No. 1 filter paper 22 × 4 inches was washed with distilled water containing 0.5% ammonia and dried for 24 hours. The paper was added to a beaker containing 20 ml. of distilled water and allowed to stand with occasional shaking for 20 minutes. The solution was filtered through a plug of glass wool and a 2-ml. aliquot of it was transferred to a colorimeter tube. Forty milligrams of phenol was added as an 80% solution of phenol in water and then 5 ml. of concentrated sulfuric acid. The absorbance of the solution was determined with an Evelyn colorimeter.

The solutions from the washed and unwashed papers showed absorbances of 0.03 and 0.06, respectively.

Procedure. Two sheets of Whatman No. 1 filter paper 8 × 22 inches are prepared as described below. One of the sheets is used as a blank. Before placing any sugars on the paper, lines are drawn as follows: Two lines are drawn lengthwise 1.5 inches from the edge of the paper. Two more lines are drawn, the first 1 inch from the top and the second 3.5 inches from the top. The sugars to be analyzed are placed on the paper along the 3.5-inch line. The two strips 1.5 inches from the edge are marking strips, which will be cut off and sprayed with *p*-anisidine or *p*-phenetidine trichloroacetate or ammoniacal silver nitrate after development of the chromatogram. The appearance of the spots marks the distance the sugars have traveled in the marking strips and the unsprayed center section. The amount of sugar added to the marking strip is not critical as long as enough is present to give a spot with the spray reagent. However, the amount of sugars added to the 5-inch center section of the paper must be accurately measured if it is desired to determine the absolute amounts of sugars as well as the relative amounts in the mixture.

A margin of at least 0.5 inch should be allowed, leaving 4 inches in the center to which a measured amount of sugar solution can be added from a micropipet.

The amount of sugar which can be added before overlapping of the spots occurs should be determined for each type of analysis. This can be done by putting graded amounts of sugar on several papers, drying, then developing with solvent, drying, and then spraying the entire paper. This will show whether the sugars move in discrete bands, and how much margin should be allowed along the edges. The larger the amount of sugars which can be added, the less significance the blank will have. In most cases about 600 to 1000 γ of sugar should be added. Dimler and others (*13*) recommend that another paper be prepared to counteract the variations in delivery that may occur with micropipets. To this paper they add standard amounts of known sugars, using the same pipet and the same technique. This procedure does not, of course, eliminate the need for a blank determination, because the presence of the soluble carbohydrate fraction in the filter paper will have a relatively greater effect at low sugar concentrations. After the sugars have been added to the paper, the chromatograms are developed for a long enough period so that the sugars to be analyzed are clearly separated. After the chromatogram has been dried in the air, the side marking strips are cut off and sprayed to show the location of the sugars in the center section. The center unsprayed portion of the chromatogram is then cut up into sections corresponding to the locations of the sugar. Each section is transferred to Petri dishes, beakers, or other suitable containers that can be covered or closed. The blank paper is cut up to correspond to the area and location of the sugars of the other paper. Twenty milliliters of distilled water is added to each of the Petri dishes, which are then covered and allowed to stand for 30 minutes with occasional shaking. During this time the sugar becomes equally distributed throughout the liquid and solid phases (water and cellulose). The eluate is filtered through glass wool and the concentration of sugars determined as described before, with the important difference that the absorbance of the blank reading is subtracted from that corresponding to the sugar before referring to the standard curve.

Results. EFFICIENCY OF EXTRACTION OF SUGARS FROM FILTER PAPER. This is illustrated by two typical experiments:

1. With a micropipet, 0.102 ml. of a solution containing 4.52 mg. of D-fructose was added to a piece of Whatman No. 1 paper (3 × 5 inches). The paper was allowed to dry in the air for 24 hours and then soaked in 20 ml. of distilled water for 0.5 hour to extract the sugar. (In another series of experiments it was found that sugars are extracted from the paper almost immediately.) The extract was filtered through glass wool and a 2-ml. aliquot of the filtrate added to 20 ml. of water. Two milliliters of this diluted solution was treated with 258.4 μl. of 80% aqueous phenol, followed by 5 ml. of concentrated sulfuric acid. The observed absorbance at 490 mμ was 0.545 and 0.538.

In a blank experiment a piece of paper of identical size was extracted for 0.5 hour with 20 ml. of water. A 2-ml. aliquot was treated with phenol and sulfuric acid as described above. The absorbance was 0.10 (average of three results). Hence, the absorbance correction for the blank $= 0.10 \times \frac{2}{22} = 0.01$.

Corrected absorbance for the sugar determination $= 0.54 - 0.01 = 0.53$. From the standard curve for fructose, an absorbance of 0.57 is equivalent to 42.4 γ of sugar. Therefore, the amount of fructose equivalent to an absorbance of $0.53 = \frac{0.53}{0.57} \times 42.4\ \gamma$. Hence the total fructose recovered $= \frac{0.53}{0.57} \times 42.4 \times \frac{20}{2} \times \frac{22}{2} = 4336\ \gamma$.

$$\text{Recovery} = \frac{4336}{4520} \times 100 = 96\%.$$

2. A similar experiment carried out with D-glucose (400 γ) added to a piece of paper (2 × 2 inches) gave a recovery of 100%. Additional experiments with D-mannose, D-xylose, and L-arabinose, and with methylated sugars such as 2,3,4,6-tetra-*o*-methyl-, 2,3,6-tri-*o*-methyl-, and 2,3-di-*o*-methyl-D-glucose with and without solvent migration using phenol-water, butanol-ethanol-water, and methyl ethyl ketone–water azeotrope gave recoveries of 95 to 100%.

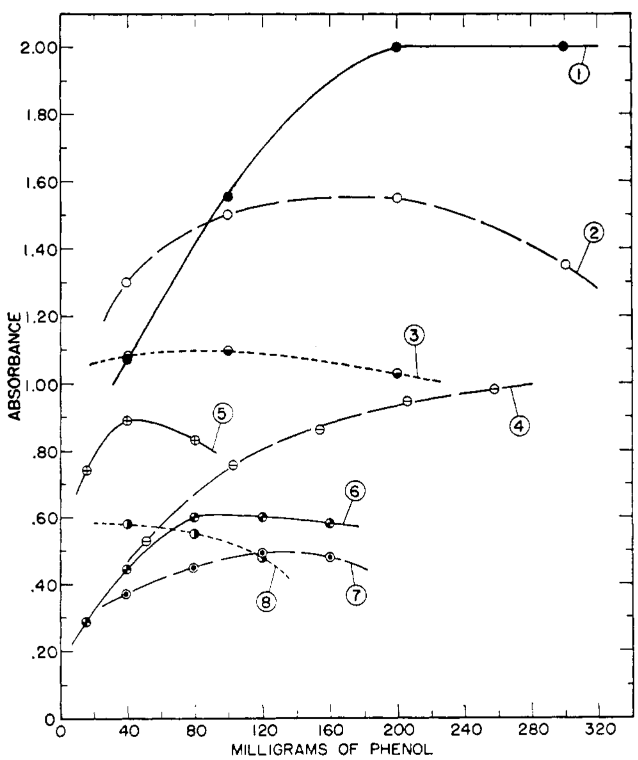

Figure 10. Absorbance *vs.* amount of phenol

1. D-Fructose, 80 γ, Beckman Model DU, 490 mμ
2. Sucrose, 80 γ, Beckman Model DU, 490 mμ
3. Raffinose, 80 γ, Beckman Model DU at 490 mμ
4. 5-(Hydroxymethyl)-2-furaldehyde, 40 γ, Beckman Model DU, 485 mμ
5. 2,3-Di-*o*-methyl-D-glucose, 80 γ, Evelyn, filter No. 490
6. 2-Furaldehyde, 20 γ, Evelyn, filter No. 490
7. 2,3,4,6-Tetra-*o*-methyl-D-glucose, 80 γ, Evelyn, filter No. 490
8. 2,3,6-Tri-*o*-methyl-D-glucose, 51 γ, Evelyn, filter No. 490

ANALYSIS OF A SYNTHETIC MIXTURE OF SUGARS. (1) A solution containing D-fructose (3.18 mg.) and D-glucose (0.20 mg.) was transferred to a piece of Whatman No. 1 paper (8 × 22 inches) as described previously. The chromatogram was developed for 24 hours by use of phenol saturated with water as the solvent. The paper was removed from the chromatographic chamber and allowed to dry for 24 hours. The marginal strips were cut off and sprayed with *p*-anisidine trichloroacetic acid reagent (small amounts of phenol do not interfere). After reassembling the chromatogram, the best line of demarcation was drawn between the two spots and the sections were cut out (glucose, 6 to 8.5 inches, fructose, 8.5 to 11 inches from the starting line), together with the corresponding blanks as previously described. The pieces of paper containing the two sugars and the two blanks were extracted and filtered. The concentration of the two sugars was then determined by the phenol–sulfuric acid reagent, reference being made to standard curves for glucose and fructose. The results were as follows:

Glucose Recovery

Absorbance of the eluate (2 ml. out of 20 ml. removed for test)	0.32
Absorbance of blank	0.10
Absorbance for glucose	0.22

From the standard curve for glucose absorbance, $0.45 = 42.4\ \gamma$ glucose

$$\text{Absorbance of } 0.22 = \frac{0.22}{0.45} \times 42.4\ \gamma \text{ glucose}$$

$$\text{Total glucose recovered} = \frac{0.22}{0.45} \times 42.4 \times \frac{20}{2} = 206\ \gamma \text{ glucose}$$

Recovery = 103%.

Fructose Recovery

Absorbance of eluate (diluted 2 ml. to 20 ml. of water)	0.40
Absorbance of blank	0.01
Absorbance for fructose	0.39

From the standard curve for fructose absorbance, $0.57 = 42.4\ \gamma$ fructose

$$\text{Absorbance of } 0.39 = \frac{0.39}{0.57} \times 42.4\ \gamma \text{ fructose}$$

$$\text{Total fructose recovered} = \frac{0.39}{0.57} \times 42.4 \times \frac{20}{2} \times \frac{22}{2} = 3200\ \gamma \text{ fructose}$$

Recovery = 101%.

(2) For a solution containing D-mannose and D-glucose, the following results were obtained:

Solvent developer	1-butanol-ethanol-water
Time, hours	48
Paper	Whatman No. 3
D-Mannose added, γ	440
D-Mannose recovered, γ	417
% recovery	95
D-Glucose added, γ	470
D-Glucose recovered, γ	440
% recovery	93.5
Glucose in original mixture, %	51.5
Glucose calculated from analysis, %	51.3

The close agreement is fortuitous, but numerous experiments with mixtures of methylated and unmethylated sugars have shown that recoveries of 100 ± 5% or better are to be expected. In the above experiment the recoveries were not so good as expected, but it is believed that this is due to the fact that the sugar bands with Whatman No. 3 are less compact than those with Whatman No. 1; for this reason the No. 1 paper is preferred.

Table IV. Wave Length *Vs.* Absorbance for Starch[a]

(Starch-phenol-sulfuric acid, Beckman Model DU, slit width 0.1 mm., 103 mg. of phenol)

Wave Length	Absorbance for 62.4 γ Starch	Absorbance for 124.8 γ Starch
410	0.21	0.42
420	0.24	0.47
430	0.25	0.495
440	0.257	0.51
450	0.29	0.578
460	0.371	0.745
470	0.52	1.03
480	0.68	1.32
485	0.735	1.42
488	0.75	1.45
490	0.75	1.45
495	0.70	1.35
500	0.60	1.15
510	0.33	0.635
520	0.197	0.383
530	0.147	0.294

[a] Baker's potato starch dried for 3 days in vacuo (30 mm.) at 75° C.

Conclusions. The phenol–sulfuric acid method can be applied to the analysis of any mixtures of sugars and their methyl derivatives that are amenable to separation by paper chromatography. Thus it has been applied to the analysis of mixtures of methyl sugars separated on paper by butanol-ethanol-water or methyl ethyl ketone–water azeotrope. The method has also proved of value for the analysis of hydrolyzates of oligosaccharides; of polysaccharides such as starch (Table IV), glycogen, plant gums, and hemicelluloses (*15*); and for the determination of the amount of sugar in urine and in blood.

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Digester and Filter for Preparing Extract Solutions from Solids—Correction

After publication of the article on "Digester and Filter for Preparing Extract Solutions from Solids" [Anal. Chem. **27**, 1669 (1955)] attention was called to an article published a short time earlier by M. Potterat and H. Eschmann [*Mitt. Lebensm. Hyg.* **45**, 329–31 (1954)], in which a design for an apparatus having substantially the same features was presented. Since receiving this information the authors have sought to learn how the earlier article escaped notice and found that because of the time factor the publication in which it appeared could not have been available to them when the manuscript was prepared.

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