SEPARATION AND QUANTITATIVE DETERMINATION OF SIMPLE SUGARS USING THIN-LAYER CHROMATOGRAPHY

by

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A Thesis Submitted to the Faculty of the

DEPARTMENT OF AGRICULTURAL CHEMISTRY AND SOILS

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

In the Graduate College

UNIVERSITY OF ARIZONA

1965

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ACKNOWLEDGMENTS

The author thanks Dr. Thomas C. Tucker for the advice, encouragement and time he has given so generously. His support throughout this research project is deeply appreciated.

Thanks are extended to Dr. Wallace Fuller and Dr. Raymond Hannapel of the author's guidance committee for their interest and comments in editing this thesis.

The capable technical assistance of Mrs. Joyce Truitt, Miss Sue Hibbs, William Taxerman and Walter Smith during various phases of this project are acknowledged.

This research was supported in part by Grant 62-38 from the Cotton Producers Institute of the National Cotton Council of America.

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ABSTRACT

Thin-layer chromatography (TLC), a new analytical tool, has found many applications in the separation and qualitative identification of small quantities of organic compounds. The simple sugars are one class of compounds for which TLC as yet has not been satisfactory.

The present work investigates possible procedures to improve the usefulness of cellulose thin-layers in separating sugars. Several solvent systems previously used in the paper chromatographic separation of sugars were evaluated with this medium.

Two colorimetric procedures previously used for the quantitative analysis of sugars separated by paper chromatography have been employed in this study in an effort to make thin-layer chromatography even more effective for the analysis of sugars.

The applicability of these methods to the field of plant physiology is demonstrated using Gossypium hirsutum var. Acala 4-42 as the test plant. The sugars present in the free state in various tissues of this plant were separated and qualitatively identified. The quantities of fructose, glucose, and sucrose were then determined for each tissue.

INTRODUCTION

Thin-layer chromatography (TLC) is a useful tool for the rapid separation of a wide range of compounds of biological interest. The classes of compounds which can be separated by TLC depend both on the sorbent material used for the layer and the solvent system selected. The majority of the workers have used silica gel for the sorbent layer with a fair degree of success. However, TLC has not been as successful for the separation of sugars as it has been for the separation of many other compounds.

Paper chromatography has been the method of choice for many years for the separation of sugars. Samples containing from 25γ to over 1 mg. of each sugar can be successfully separated. The major disadvantage of paper chromatography is that the time necessary to separate a series of sugars ranges from 24 hours to three or four days. When many samples must be analyzed this may become a serious problem.

Innumerable methods have been developed for the quantitative determinations of sugars. Colorimetric procedures are the most applicable when working in the microgram range. Many of these methods have been developed using pure sugars. When working with plant extracts, other compounds often co-chromatograph with the sugars and interfere with the quantitative methods.

The aims of the present investigation are firstly, to develop a method for the separation of simple sugars which will combine the separation characteristics of paper chromatography with the speed of TLC. Since cellulose is available for TLC this will be used for the sorbent.

Secondly, a quantitative method will be adapted for use with TLC. Finally, cotton plant tissue will be used as a representative plant source to determine the applicability of the method to the separation and determination of sugars in the presence of other compounds ordinarily present in the biological matrix.

LITERATURE REVIEW

Thin-layer chromatography is an analytical tool which is gaining wide acceptance. The historical development, theoretical aspects, and several applications have recently been discussed (31, 24, 7). Although many different sorbents are available which are suitable for TLC, the majority of the workers have used silica gel with a binder for the sorbent layer. One group of compounds for which silica gel has not been a satisfactory medium is the naturally occuring free sugars. Poor separation of some of the more common sugars and the low capacity of the plates are two disadvantages in this application (32).

Paper chromatography has proven quite useful for the separation of small quantities of many compounds. Theory, techniques, and applications have been reviewed by several authors (4, 30, 15). Linksens' text deals strictly with the application of these techniques to investigations in plant science (19). Although the simple sugars have been separated for many years using paper chromatography, major disadvantages are the long elution times required for satisfactory separations, the associated problems of temperature fluctuation, and the total time and equipment involved if a large number of samples are to be analyzed (5).

Using cellulose as the sorbent in thin-layer chromatography should provide the advantages of paper chromatography without its disadvantages and the advantages of TLC without the problems involved with silica gel. Randerath has found cellulose-TLC superior to paper in the separation of nucleotides (25). The author is aware of only one paper in

the literature dealing with the separation of a few simple sugars using cellulose plates (27).

Very few efforts have been made to quantitize TLC. Only one paper has dealt with the quantitative determination of simple sugars separated by TLC (23). That procedure requires 300 to 400γ of sample while the silica gel plates have a capacity of about 40γ

The colorimetric method of Dubois, et al., (12) which has found wide use and acceptance in paper chromatographic techniques is unsatisfactory for cellulose-TLC due to the persistent high background which can not be eliminated by prewashing the plates (29). Dische has recently reviewed several colorimetric procedures which are applicable to paper chromatography (10). The reaction of anthrone with carbohydrates was first described by Dreywood (11). Since then several authors have reported the application of this reaction to the quantitative determination of various carbohydrates (17, 21, 22, 28). Both Koehler (17) and Bonting (8) comment on the rate of color development with fructose in comparison to that of glucose.

Although the p-aminobenzoic acid colorimetric method as modified by Leopold has not been studied as thoroughly it will also be considered here (18).

EXPERIMENTAL

I. Preparation of Plates and Qualitative Separation

(A) Preparation of plates

The plates were washed with a detergent, rinsed well with tap water, followed by distilled water and finally methanol (reagent grade).

The cellulose slurry was prepared as follows: 15 gm. of cellulose 300 MN (Macherey, Nagel & Company) were mixed with 90 ml. of a deionized water-methanol solution (5:1, v/v) by adding small portions of the solution to the powder and stirring well. A homogeneous slurry resulted. This was sufficient to cover five 20x20 cm. plates and two 5x20 cm. plates. A 0.37 mm. thick layer was applied with an adjustable applicator (Brinkman-Desaga).

The plates were always dried in a hood for two hours and then stored in a desicator cabinet overnight before use. (The relative humidity in the laboratory seldom was over 15 percent).

(B) Sample preparation

The sugar solutions were made by dissolving 1 mg. of sugar in 10 ml. of 10 percent isopropanol. A mixture of glucose, fructose, and sucrose was prepared in the same way. The following sugars were studied:

Disaccharides: sucrose, lactose, cellobiose, maltose

Aldohexoses: a-D-glucose, D-mannose, D-galactose

Ketohexoses: D-fructose, L-sorbose

Aldopentoses: D-arabinose, L-arabinose, D-xylose, D-lyxose, D-ribose.

(C) Solvents

The following solvents were evaluated:

1. Formic acid-methyl ethyl ketone-tert. butanol-water (15:30:40:15, v/v) (13).

2. Ethyl acetate-pyridine-water (2:1:2, v/v) (16).

3. Ethyl acetate-isopropanol-water (65:24:11, v/v) (33).

4. n-Butanol-acetic acid water (6:3:1, v/v) (33).

5. Methyl ethyl ketone-acetic acid-methanol (3:1:1, v/v) (33).

6. Ethyl acetate-acetic acid-water (3:2:3, v/v) (6).

7. n-Butanol-pyridine-water (45:25:40, v/v) (6).

8. Isopropanol-pyridine-acetic acid-water (8:8:1:4, v/v) (14).

9. Phenol aq. (ca. 90%) -water (10:1.25, v/v) + 0.002% 8-hydroxyquinoline (35).

(D) Detection reagent

The detection reagent used was 2-aminodiphenyl - oxalic acid dissolved in 85 percent ethanol (14). This reagent locates disaccharides as well as hexoses and pentoses. The 2-aminodiphenyl is no longer commercially available because of its suspected carcinogenic properties. Several methods of preparation are given in the literature (1,26).

(E) Chromatographic procedure

The samples were spotted at 1 cm. intervals using a micropipette. The pentoses were applied at 30γ per spot and the others at 40γ per spot. The origin was 2 cm. above the cotton edge of the plate. The film was broken 15 cm. above the origin. The film was also broken vertically 0.5 cm. from each side to eliminate edge effects (9). Solvent was placed in the tank 15 minutes before the plates were added. The laboratory

temperature was 23 C.

After the plates were developed and dried they were sprayed with the detection reagent. The sugars were located by heating the plates with an industrial hot air drier. A lighter background resulted by this method than when heated for 10 minutes in an oven at 110 C.

II. Quantitative Analysis of Standard Sugars

(A) Separation of mixtures

Plates were prepared as described above. Samples were spotted at 1.5 cm. intervals across the origin, leaving the first and last spaces blank. The plates were developed two times in solvent 1 described in the preceeding section.

(B) Location of sugars and removal from plate

After the plate has been developed and dried the plate was masked using clean glass plates leaving only the samples nearest the edges exposed. These areas were sprayed with the detection reagent and heated with the hot air gun to develop the colors. The plate was then sectioned horizontally and vertically by breaking the cellulose layer with a sharp pencil. Each square contains one sugar. The indicator strips were not used in the quantitative procedures. Blanks were taken from the edges at the same height as the sugars. Figure 1 shows a plate ready for scraping. Each square was scraped into a clean 10 ml. Pyrex culture tube using a sharp single-edged razor blade. The tubes are then capped until needed.

(C) Reagents

1. Eluents

95% acetone (reagent grade, v/v in deionized water).

Deionized water

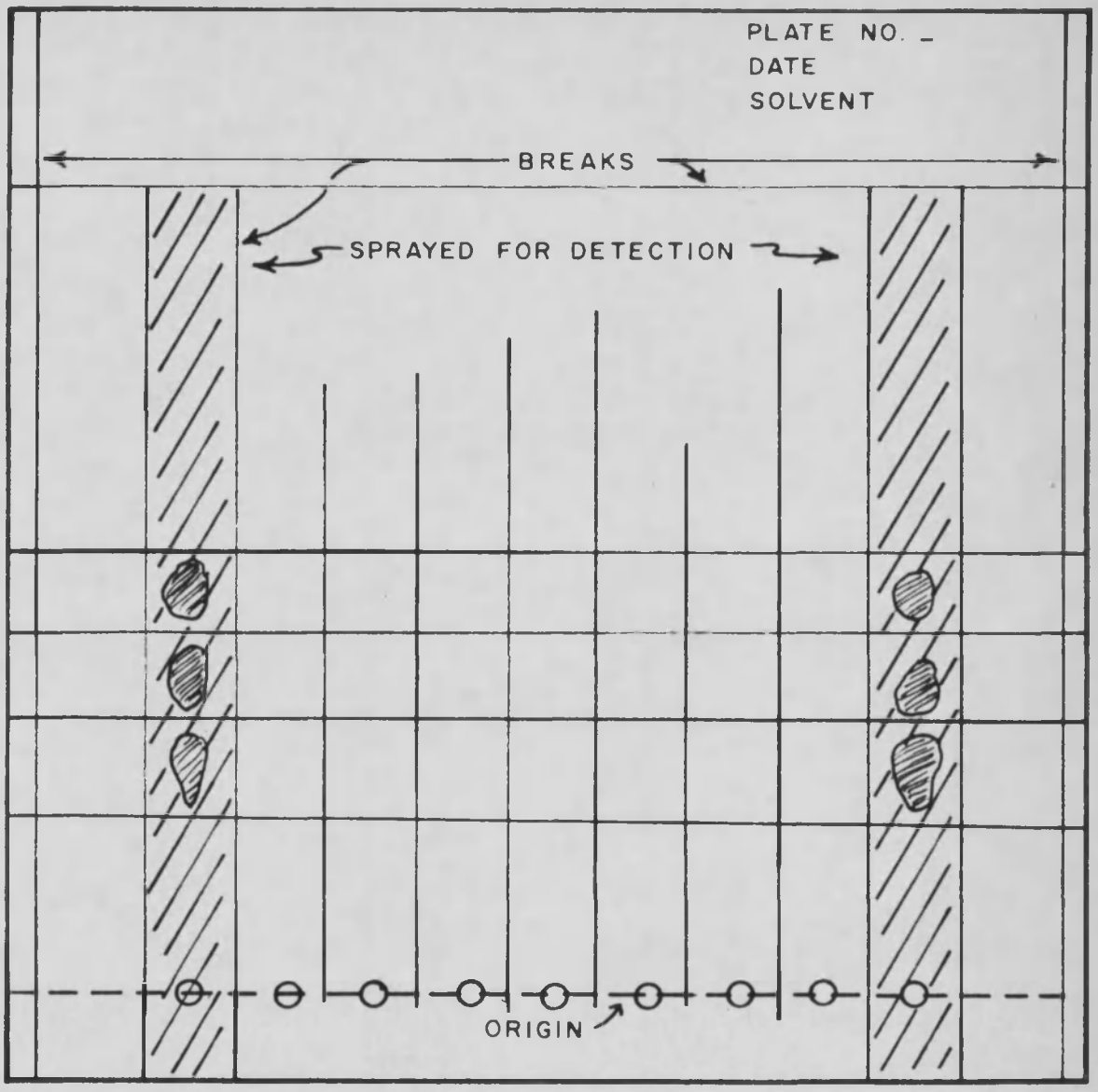


FIGURE 1. TLC PLATE DEVELOPED AND READY FOR SCRAPING.

2. p-aminobenzoic acid procedure

1.5% p-aminobenzoic acid (Eastman) in glacial acetic acid (J. T. Baker Co., reagent grade, w/v).

1.3% phosphoric acid in glacial acetic acid (J. T. Baker Co., reagent grade; 1.3 ml. concentrated (87%) H_3PO_4 per 100 ml.).

3. Anthrone procedure

2% anthrone (Eastman) dissolved in ethyl acetate (reagent grade, w/v). (20).

Concentrated sulfuric acid (96 $\pm$ 0.7%) (reagent grade).

(D) Quantitative procedure using p-aminobenzoic acid

To each tube of scrapings 5 ml. of 95 percent acetone were added by buret. The tubes were covered with Parafilm and shaken vigourously to break up the cellulose mat. The tube was then gently shaken to wash the cellulose flakes to the bottom of the tube. The tube was set aside until most of the cellulose had settled.

Each sample is then filtered with suction through a medium-porosity Hirsch filter into a clean tube (Figure 2). The samples are taken to dryness in a vacuum oven at 60 C and 90 mm. Hg.

Using microburets 0.25 ml. deionized water, 2.0 ml. p-aminobenzoic acid reagent, and 2.0 ml. phosphoric acid reagent were successively added to the dry sample. The tubes were covered with Parafilm, aggitated gently and placed in a boiling water bath for one hour. They are cooled for at least 45 minutes. The absorbance is determined on a Beckmann DU spectrophotometer at 365 mu using a slit-width of 0.2 mm.

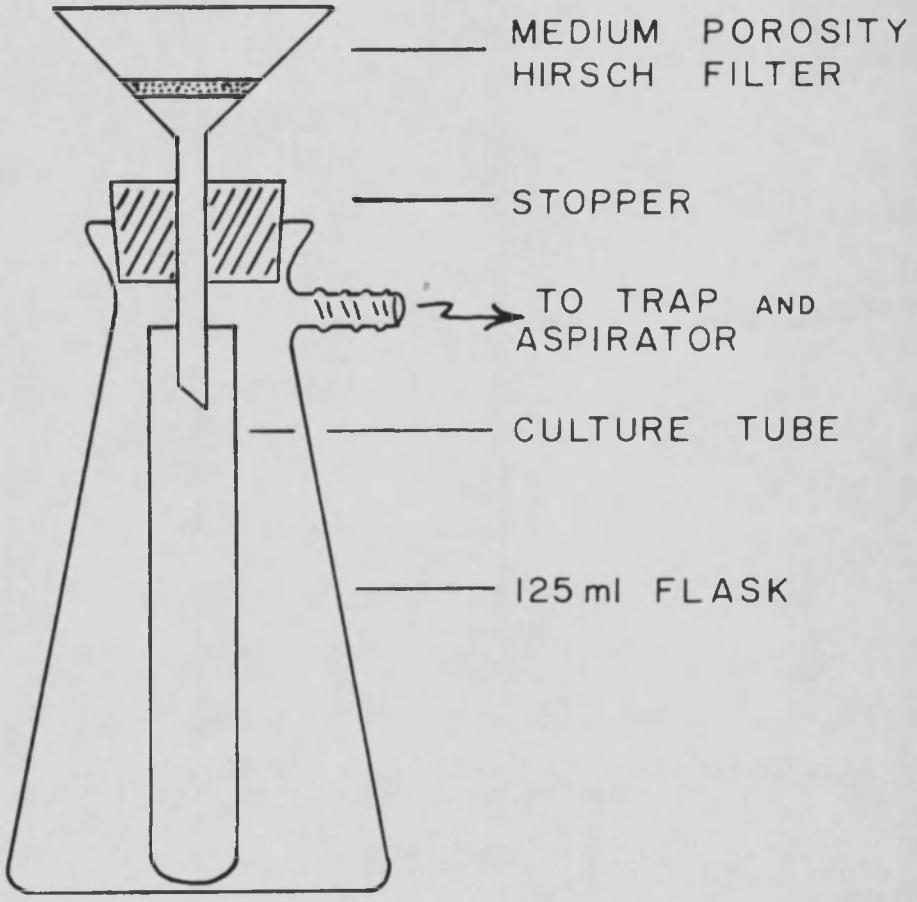


FIGURE 2.

FILTRATION ASSEMBLY

(E) Quantitative procedure using anthrone

The samples were eluted with 5 ml. of 95 percent acetone, filtered, and dried as described above. The dried samples were placed in an ice-water bath and 2 ml. water, 0.5 ml. anthrone reagent, and 4 ml. concentrated sulfuric acid were added in order. The tubes were covered with Parafilm, shaken gently while cold to achieve partial mixing, and then the tube was inverted ten times to mix the reagents completely. CAUTION!

Tubes containing ketohexoses such as fructose were then heated in a 60 C. bath for five minutes. Tubes containing aldohexoses such as glucose, or dissacharides such as sucrose, were heated in an 80 C. water bath for thirty minutes. After the proper heating the tubes were removed from the bath and cooled to room temperature by placing them in a room-temperature water bath for a short time. The absorbance was then determined on a Beckmann DU at 625 mu (blue tube) and a slit-width of 0.1 mm.

III. Determination of Sugars in Cotton Plant Tissue

A weighed smmple of dry, finely ground plant material was extracted with 80 percent ethanol. The extraction may be done either with a Soxhlet extractor or with a high speed homogenizer. The extract was concentrated to a known volume and centrifuged to remove any solids present.

Using a micropipette, a sample was applied to the TLC plate and the sugars isolated in the usual manner.

The particular plant material used in this study was Gossypium hirsutum var. Acala 4-42. The plants were field-grown south of Tucson during 1963. The particular plant sample was taken August 16, 1963.

The plant was separated into root, stem, and leaves and dried in a forced-draft oven at 70 C. for four days. The dry material was ground to pass a 40-mesh screen. Soxhlet extractions were carried out for three days per 3 gm. sample. The cooled extracts were concentrated to 10 ml. and refrigerated until used.

RESULTS AND DISCUSSION

I. Qualitative Separation of Simple Sugars

In terms of solute detectability and time, it is advantageous to find a solvent or solvents which will give good separation of sugars in one dimension. Since cellulose thin-layer plates possess the same partition properties as paper, it seemed advisable to evaluate several solvents previously used for the separation of sugars by paper chromatography, as well as a few solvents used with silica gel TLC, to determine their applicability for the separation of sugars on cellulose layers.

Nine solvents were evaluated in this study. Special emphasis was placed upon the property of the solvent to separate sucrose, glucose, and fructose in one dimension without a long period of pre-saturation of the plate. These sugars are of principal interest because they are the most commonly occurring free sugars in higher plants.

These nine solvents ascend more slowly on cellulose layers than on silica gel layers. The development time is still rapid when compared to the usual developing times of 24 hours or longer for paper chromatography. A slightly faster development time can be achieved using cellulose with $CaSO_4$ as a binder. No other differences were observed in this laboratory between cellulose with and without a binder.

Table 1 lists the characteristic colors produced by the spray reagent. The order of appearance and relative intensities are also given. These results were the same for all solvents tested.

Table 1. Characteristic colors of sugars sprayed with 2-aminodiphenyl.

Class	Color	Order of appearance	Relative intensity
Disaccharides	Light tan	3	3
Aldohexoses	Very dark brown	2	2
Ketohexoses	Green, to greenish-brown on prolonged heating	4	4
Aldopentoses	Red	1	1

Table 2 lists the $R_f x100$ values for the various solvents. The $R_G x100$ values for solvent 1 are also given. In this case, the plate was developed twice in the same direction. Several of the solvents gave severe streaking, especially the more volatile, faster running solvents. In some cases this made it impossible to determine an R-value.

Solvent 1 is the prefered solvent. The spots containing pentoses enlarged to about twice the diameter of the original spot after being developed twice. The higher molecular weight sugars diffused even less. All spots were nearly circular with no bearding or tailing, whereas for all of the other solvents tested the spots were elongated and bearded (streaked), and there was an occasional double spot. Figure 3 shows the results of a 15 cm. development using solvent 3.

Since the R_f values are low in solvent 1 multiple development can be used to advantage (34). Figure 4 shows the separation achieved after two developments in the same direction using solvent 1.

The literature to date indicates that the use of TLC for the separation of sugars is inferior to paper chromatography because of the small

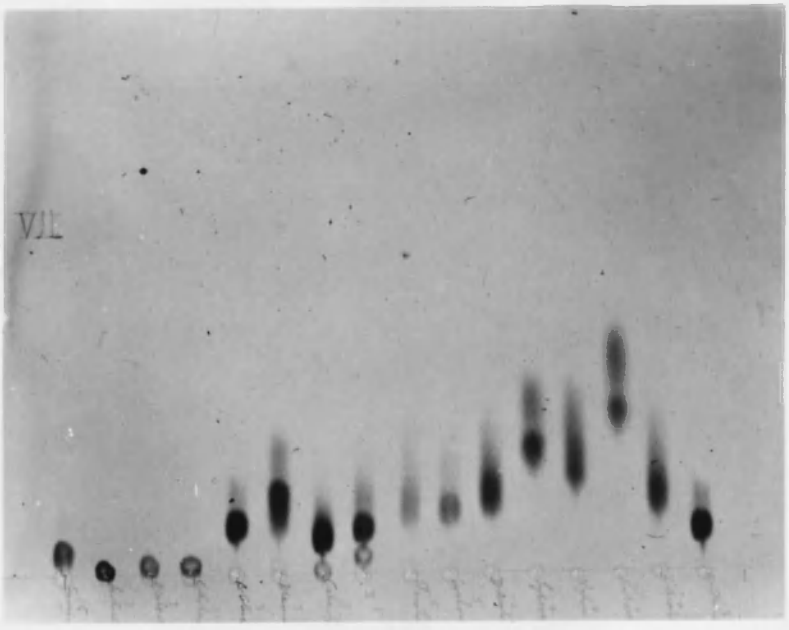
Table 2. R-values of sugars in nine solvents on cellulose.

	R_fx100									R_Cx100
Compound/Solvent	1	2	3	4	5	6	7	8	9	1
Sucrose	10	20	4	S	0	63	40	67	37	65
Lactose	4	17	1	5	0	56	31	39	37	26
Maltose	6	S	2	7	0	62	34	48	34	38
Cellobiose	5	27	1	4	0	60	33	45	32	32
a-D-Glucose	19	S	S	17	0	63	40	47	35	100
D-Mannose	23	40	S	S	0	65	43	60	40	123
D-Galactose	17	S	S	S	0	60	37	53	40	91
D-Fructose	24	S	1	22	0	62	41	61	47	130
L-Sorbose	23	S	10	20	0	63	41	60	37	123
D-Arabinose	27	40	S	23	0	63	41	61	50	145
D-Lyxose	33	48	S	S	0	67	46	57	46	170
L-Lylose	30	47	S	25	0	67	46	65	41	160
D-Ribose	39	53	S	S	0	69	49	69	57	191
L-Arabinose	29	39	S	23	0	63	40	60	51	151
Mixture: a-D-Glucose, D-Fructose, Sucrose	Y	N	N	N	N	N	N	N	Y	Y
Approximate time (hrs.) for solvent to travel 15 cm.	3	2	3	4	½	3	4	4	6	5 Total time 2 developments

N = No separation of mixture; Y = Yes, complete separation achieved;
S = Badly streaked, couldn't measure an R-value.

Figure 3. A 15 cm. development in ethyl acetate-isopropanol-water showing typical bearding and poor separation. From left to right the sugars are: sucrose, lactose, maltose, cellobiose, D-glucose, D-mannose, D-galactose, mixture, D-fructose, L-sorbose, D-arabinose, D-lyxose, L-xylose, D-ribose, and L-arabinose.

Figure 4. The separation of sugars achieved after two developments of 15 cm. in the formic acid-methyl ethyl ketone-tert. butanol-water solvent. The sugars are in the same sequence as in Figure 3.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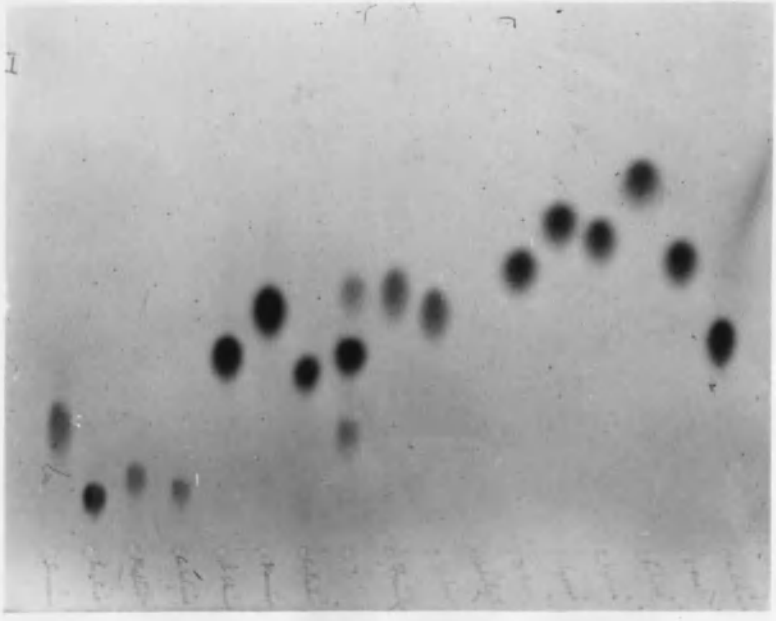
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quantities of sugars that can be used. These results were obtained on silica gel. With the solvent 1 - cellulose substrate combination, mixtures containing 100γ or more of sucrose, glucose, and fructose can be separated using the multiple development technique. The upper limits for the separation of most pentoses appear to be 50γ.

Galactose can be separated from glucose using this system. After two developments with solvent 1, about 15γ of galactose can be separated from the same quantity of glucose. If larger amounts of these sugars are present, three or more developments may be required for complete separation. With the phenol-water system (solvent 9)galactose moves farther than glucose, permitting a satisfactory separation of as much as 50γ of each sugar in one development.

Using solvent 1, the sugars are separated into classes, i.e., trisaccharides nearest the origin, then disaccharides, and so forth.

II. The Quantitative Methods

(A) p-Aminobenaoic acid method

The author was unable to find any reference to the chemistry of this procedure. The reaction will not proceed stoichiometrically with ketohexoses such as fructose, indicating an aldehyde group is necessary. This is also observed with dissaccharides such as sucrose where the absorbance values are at least 50 percent less than for an equal weight of glucose.

Ultra-violet spectra were obtained for both the sucrose and glucose products of the reaction to ascertain the wavelength chosen for the colorimetric procedure as reported in the literature was optimum (18).

The spectra were obtained on a Perkin-Elmer 202 ultra-violet - visible recording spectrophotometer using silica cuvettes and the reagent blank as the reference. The spectra of a Pyrex cuvette containing distilled water, using distilled water in a silica cuvette as the reference, was obtained to be sure that the Pyrex cuvettes could be used in routine analyses. These spectra are shown in Figure 5.

The author found that all sources of glacial acetic acid are not equally effective for this procedure. Figure 6 shows calibration curves obtained for three sources of acetic acid. The acid from Mallinckrodt and DePont both assayed at 99.5 percent minimum purity. That from J. T. Baker Co. had a reported assay of 99.8 percent minimum acetic acid. The reason for the poor results obtained using the DePont acid is unknown. Acetic acid from J. T. Baker was used throughout this project because of its ready availability.

Leopold (18) used 0.5 ml. of water in his procedure. However, it was found that 0.25 ml. water increased the sensitivity of the procedure slightly.

Reproducible values are not obtained from plate to plate. However, within a plate the results of duplicate samples will approach a straight line. Calibration curves obtained by taking the average of duplicate samples are shown in Figure 7. From the data it can be seen that standards must be run on each plate. If two concentrations of a standard mixture are applied, such as 20 and 50γ, this will be adequate to determine the slope of the line for that plate.

The color complex formed is stable for at least three hours after removal from the water bath. After this time crystals begin to form which could interfere when determining absorbance.

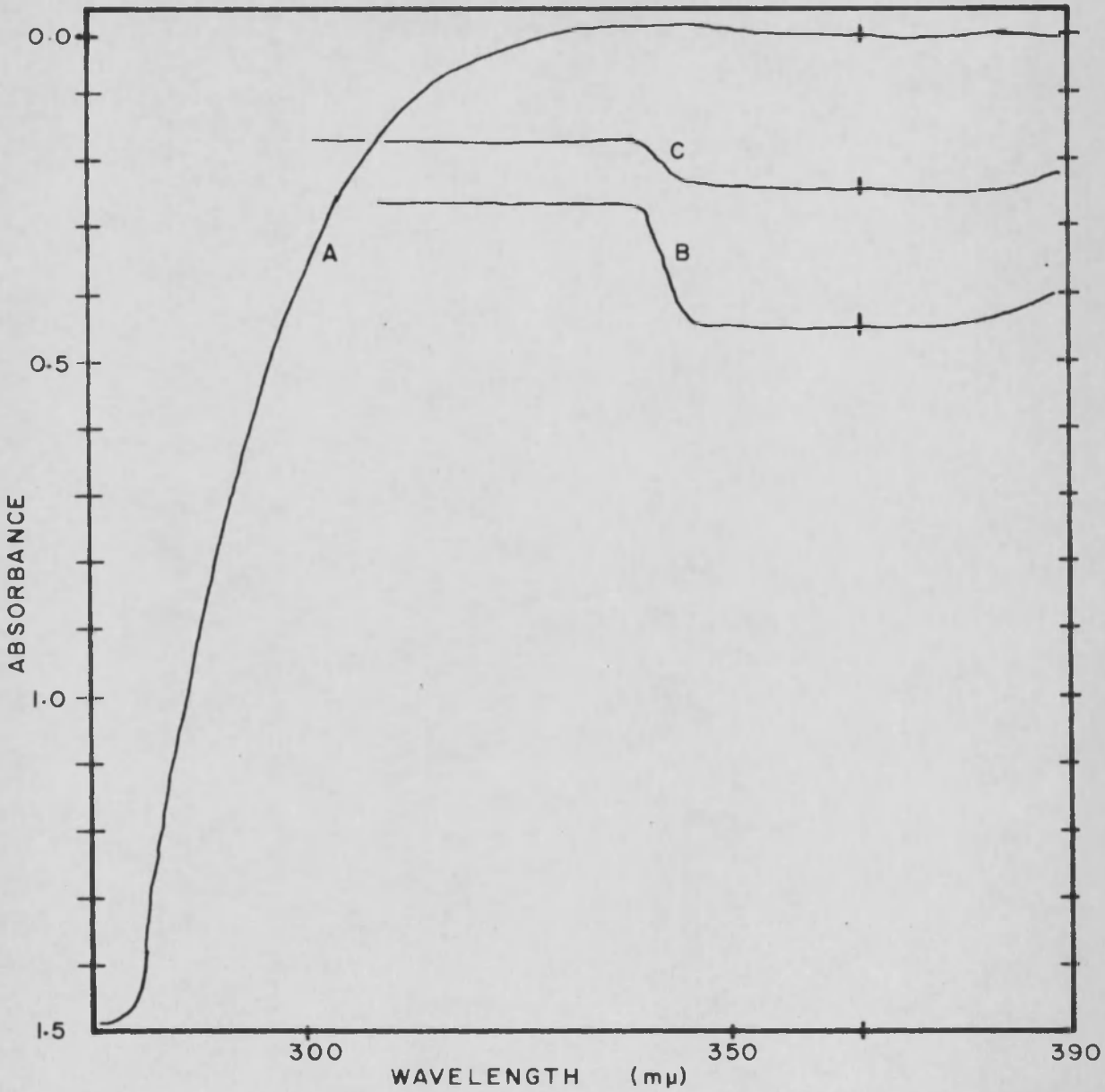

FIGURE 5. ABSORPTION SPECTRA OF SUGAR – p-AMINOBENZOIC ACID COMPLEX

A = PYREX CUVETTE vs. SILICA
B = GLUCOSE –
C = SUCROSE –

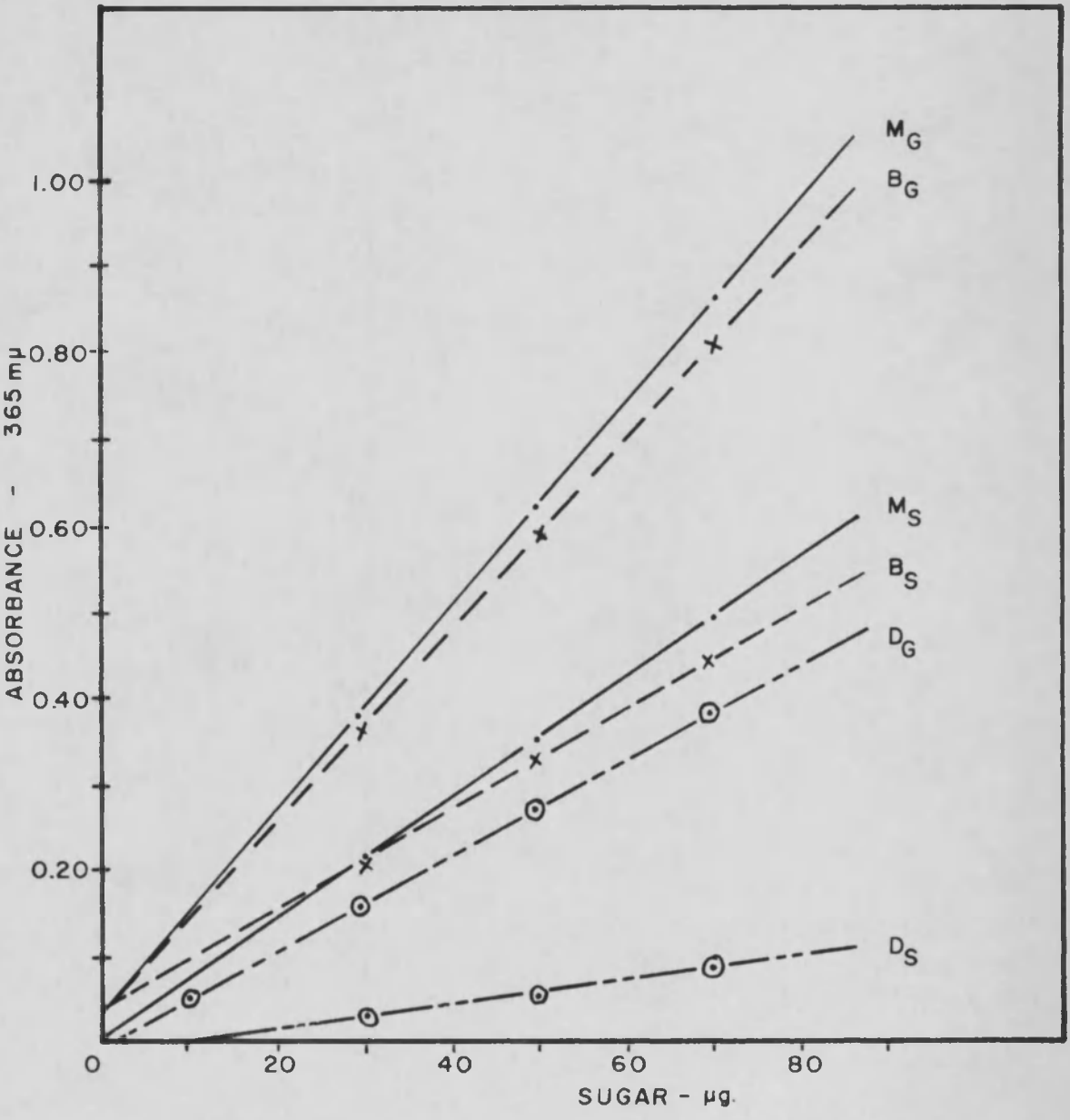

FIGURE 6. CALIBRATION CURVES USING THREE SOURCES OF ACETIC ACID.

M = MALLINCKRODT B = BAKER D = DUPONT

G = GLUCOSE S = SUCROSE

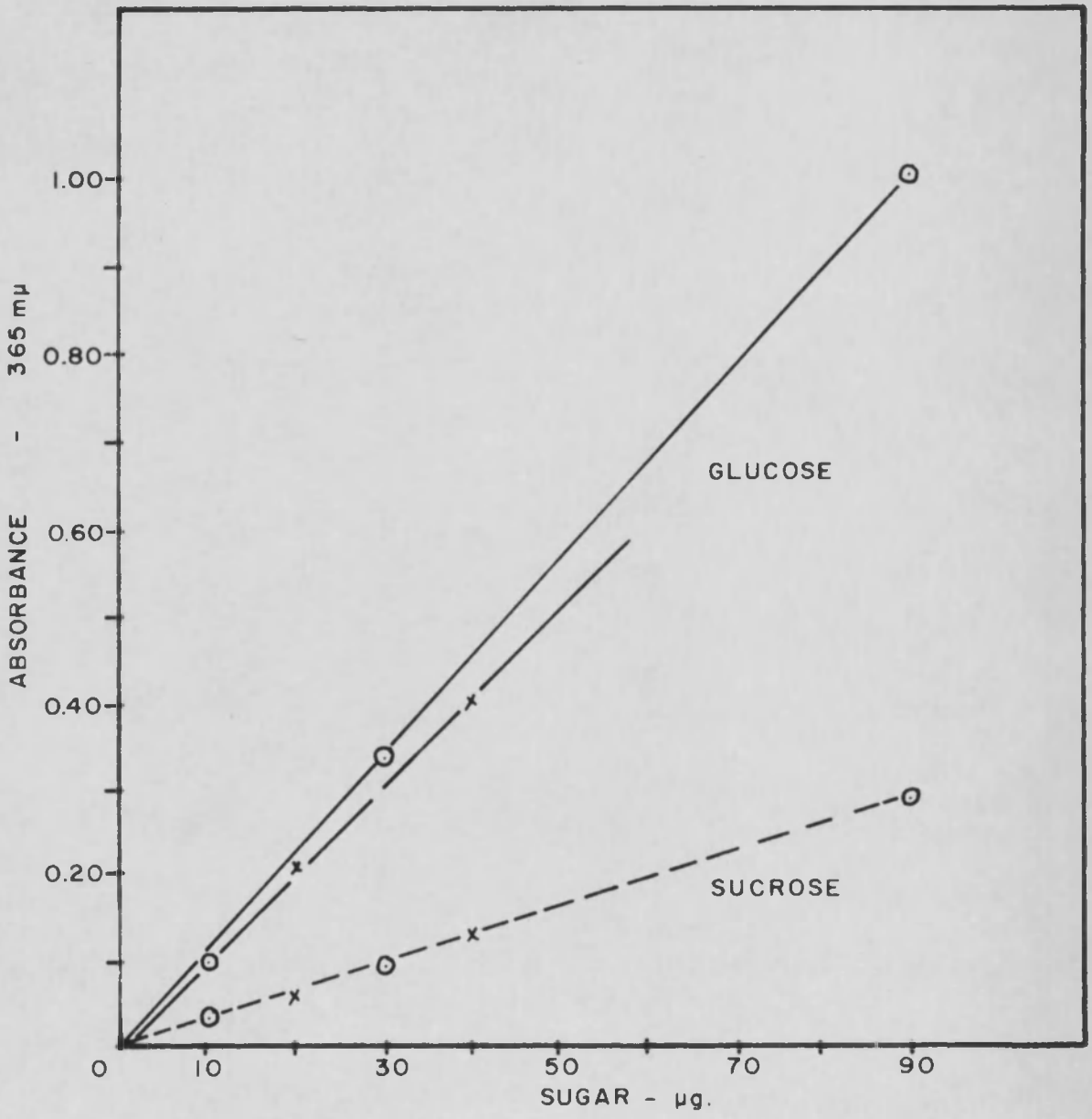

FIGURE 7. CALIBRATION CURVES FOR GLUCOSE AND SUCROSE FROM DIFFERENT PLATES. AVERAGE OF DUPLICATE SAMPLES.

⊙ = PLATE 1 X = PLATE 2

The p-aminobenzoic acid reagent is stable for at least two days. However, it is advisable to make up the reagent daily.

It is of the utmost importance that the tubes are absolutely clean and free from cellulose dust and lint. These are readily broken down to glucose under the conditions of the method.

(B) Anthrone method

The color produced in this procedure results from the reaction of the anthrone with the furfural derivative of the sugar in strong acid (2).

The absorption spectrum of the fructose-anthrone complex was obtained using the Perkin-Elmer 202 recording spectrophotometer. This spectrum is shown in Figure 8. The maximum absorption occurs at 625 mu.

A study was made of the length of time required to attain maximum absorption at different temperatures. In general agreement with Koehler (17) it was found that the anthrone-fructose complex develops maximum color in 5 minutes at 60 C. At 86 C, using concentrated sulfuric acid, the time was shortened to less than two minutes. The glucose complex reached a maximum in 30 minutes at 80 C. Sucrose essentially reached a maximum in 15 minutes at 80 C. which was stable for several minutes at this temperature. Using more dilute acids, i.e., 86 percent and 76 percent sulfuric acid, the time necessary to develop a maximum color was much longer. Also the maximum was less with the more dilute acids. These results are shown in Figure 9. (96% H_2SO_4 was used throughout this study). A direct comparison of the actual values obtained for a given concentration of sugar obtained by various investigators cannot be made since much of the older work was done on a Klett-Sommerson colorimeter rather than on a DU. Also, the investigators used different heating temperatures and different acid strengths.

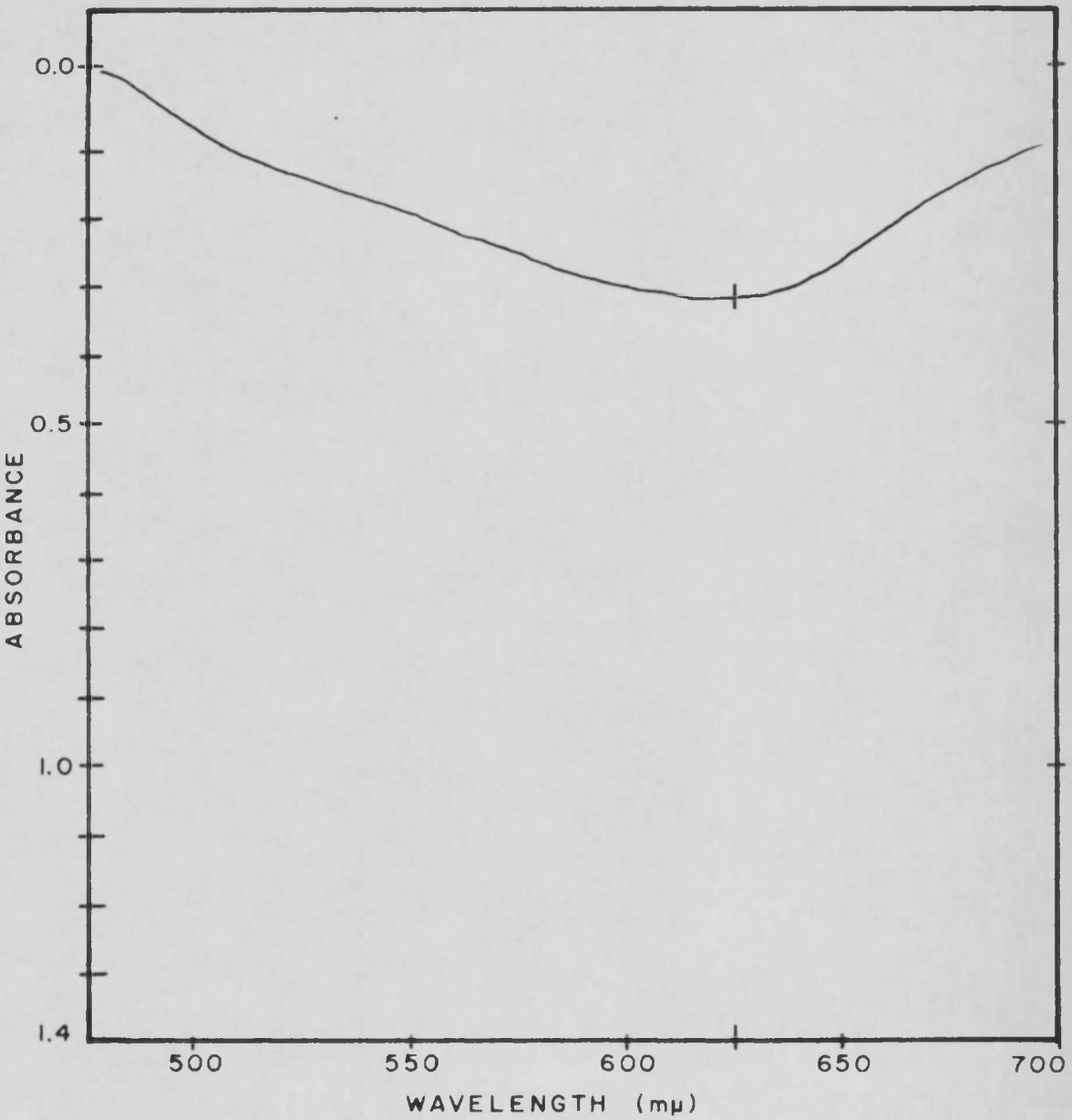

FIGURE 8. ABSORPTION SPECTRUM OF FRUCTOSE-ANTHRONE COMPLEX vs. REAGENT BLANK.

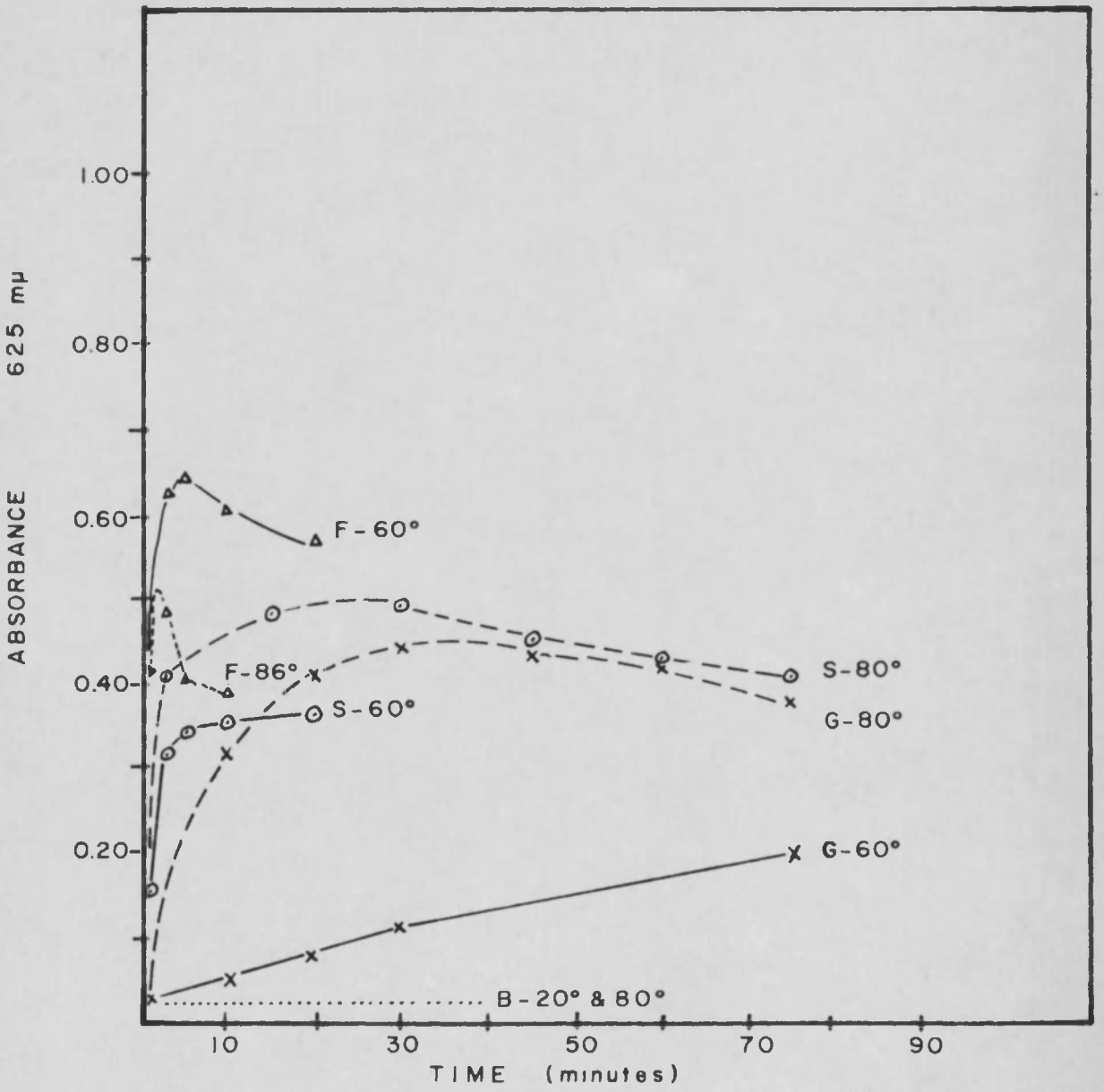

FIGURE 9. EFFECT OF TEMPERATURE AND LENGTH OF HEATING ON ABSORBANCE, 50 μg SUGAR.

F = FRUCTOSE
G = GLUCOSE
S = SUCROSE

When eluting the sample from the cellulose plate it was found preferable to use 95 percent acetone as the eluent. When water was used a very high background reading was obtained and the eluent was difficult to filter with complete recovery of the sample. When using acetone the oligosaccharides present in the cellulose are insoluble and the plate blank has the same absorbance value as a pure reagent blank. The acetone must be removed before developing the color, however, If acetone is present a dark brown color results instead of a clear green. The former is not quantitative in relation to the amount of sugar present.

The calibration curves obtained using averages of duplicate samples eluted from a cellulose plate are shown in Figure 10. These samples were eluted with water resulting in the high blank value from the plate. The instrument was zeroed against distilled water. As with the previous method the reproducibility between plates is not good. The reproducibility between samples on a given plate, however is very good. Thus, standards must be run on the same plate as the unknowns to eliminate this plate variation.

The most reproducible absorbance values are obtained using reagent not more than two days old. Usually the reagent was made one day and used the following day.

III. Free Sugars in Various Tissues of Gossypium hirsutum var. Acala 4-42

The free sugars are those not bound to other groups such as phosphate, uracil diphosphate, pigments, etc. In actual practice a small fraction of the free sugars extracted were phosphorylated but underwent autohydrolysis by the plant phosphatases during the preparation of the tissue (3). The contribution of hydrolyzed sugar phosphates to the total free sugar concentration is generally quite small.

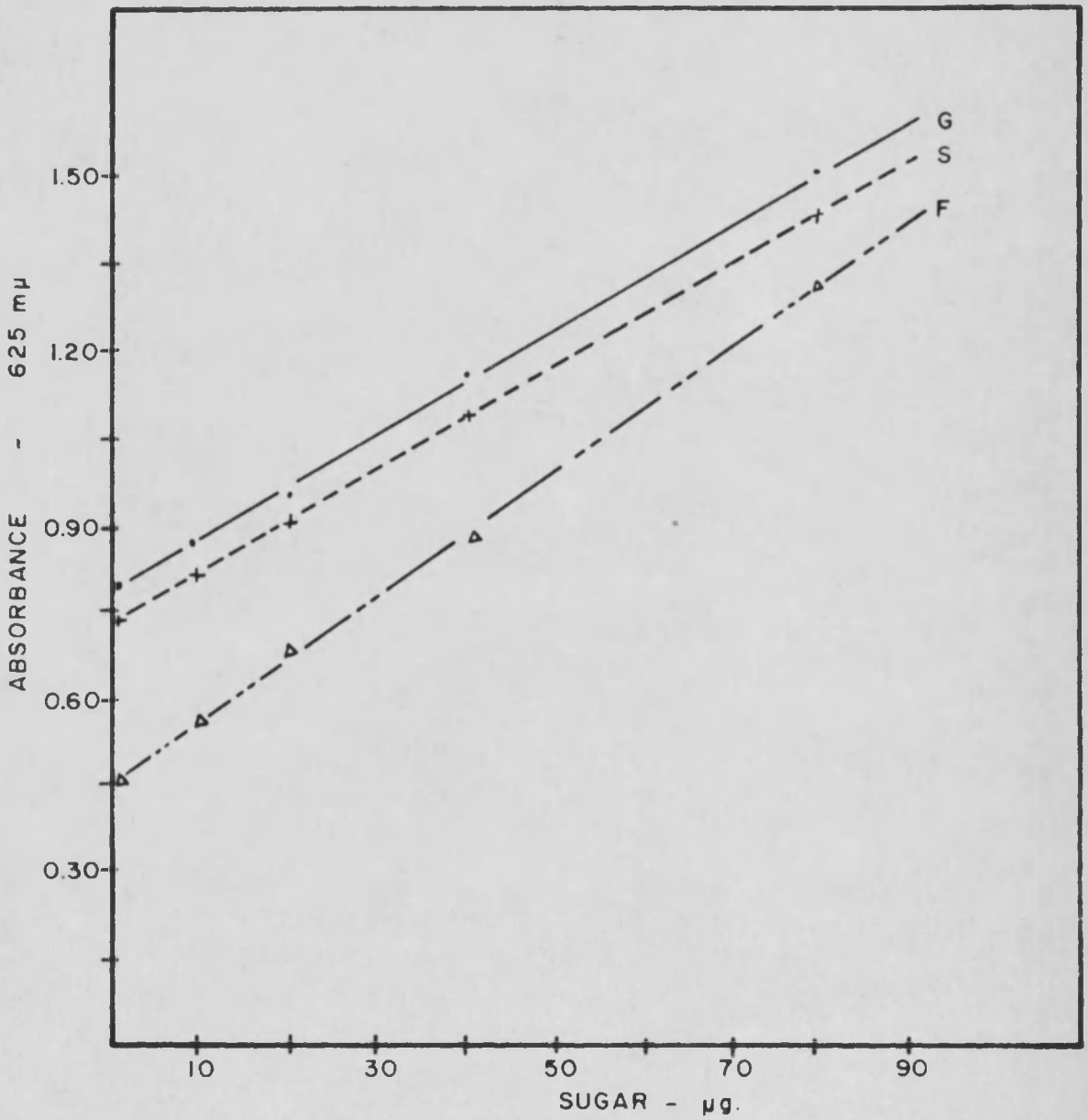

FIGURE 10. CALIBRATION CURVES FOR SUGAR–ANTHRONE COMPLEXES vs. WATER BLANK. SAMPLES ELUTED WITH WATER.

G = GLUCOSE
S= SUCROSE
F = FRUCTOSE

The free sugars present in the various parts of the cotton variety studied were identified by co-chromatographing an aliquot of plant extract with pure sugars. A visual comparison of location and color of the spots were made as well as a comparison of $R_G \times 100$ values. The comparison of these values for three solvent systems is given in Table 3. No pentoses were found, either in young plants (six leaf stage, grown in greenhouse) or in mature fruiting plants taken from the field.

Table 3. Qualitative Identification of Sugars Present in Cotton Plant Tissue

	$R_G \times 100$		
Compound / Solvent	1	9	10
Sucrose	64	133	92
Leaf X	62	117	92
Glucose	100	100	100
Leaf Y	97	97	108
Fructose	128	170	113
Leaf Z	128	150	120

Solvent 10: n-Butanol-acetic acid-water (2:1:1, v/v) (13).

Similar results were obtained for stem and root tissue.

Table 4 shows the concentrations of free sugars found in the root, stem, and leaves of the test plant as found by the procedures described above.

Table 4. Quantities of Sugars Found in Cotton Plant Tissue.

Compound / Tissue	Root	Stem	Leaf
	(mg. sugar/gm. dry tissue)		
Fructose	5.3	5.6	0.0
Glucose*	5.7	9.8	1.7
Sucrose*	14.2	6.6	1.9

* determined using p-aminobenzoic acid method.
All values are averages of duplicate samples.

The plants used for this study were taken from the field approximately one week after the period of peak flowering. The majority of the metabolites being produced by the plant at this stage of growth are being used for the production of the fruit, i.e., the seeds and lint of the cotton boll. Thus, one would not expect to find a large excess of sugars present in the free state in the leaves at this stage of plant growth. The stems included the branches and petioles, therefore most of the free sugars found in this tissue were probably being translocated to the bolls, rather than being stored before incorporation into the structural polysaccharides.

To determine if there were compounds migrating to the same positions on the plate as the sugars which might interfere with the determinations, plant samples were applied at two concentrations. In all cases the sugars recovered were in the same ratios as the quantities of extract applied. The same results were obtained when a standard addition of pure sugar was added to one of the plant samples. It is thus concluded that there are no compounds present in sufficiently high concentrations, relative to the sugars present, to interfere with any of these procedures.

If, when studying other plants, it is found that some plant constituents are interfering, either the extract can be passed through small ion exchange columns to remove ionic compounds, or different solvent systems may be used.

CONCLUSIONS

When the thin-layer plates are prepared with care to insure a layer of uniform thickness, and when the sample is applied as a small spot 0.3 cm., or less, in diameter, good reproducibility of R-values can be obtained. In some plant samples other compounds may be present in the extract in such large amounts that they will retard the migration of the sugars. Erroneous conclusions in identification may be drawn in such a case if only one solvent system is used.

The methods described provide a means for the rapid qualitative identification of sugars present in a biological extract. These sugars may then be determined quantitatively using one of the described procedures. Table 5 compares the relative sensitivities of the two methods for quantitative analysis. The anthrone method is to be preferred. In addition to

Table 5. A Comparison of the Sensitivities of the Quantitative Methods

Compound / Method	p-aminobenzoic acid	anthrone
	(Change in absorbance units per microgram sugar)	
Fructose	0	10
Glucose	11	8
Sucrose	3	8

being more widely applicable, this method is faster, requires fewer reagents and the volumes added are not as critical as with the other method. The precision of the anthrone method is 0.005 absorbance units difference between duplicate samples at the 10γ level as averaged from four plates. At the 50γ level the range is about 0.025 absorbance units as calculated on

the same basis. The p-aminobenzoic acid method gives about the same precision.

A major disadvantage to either quantitative procedure is that absolutely no contamination by cellulosic material can be permitted. A second disadvantage is that scraping the plates is a tedious procedure.

Contamination is probably the major reason for large discrepancies between duplicate samples. The second most probable reason for error is in the quantity of extract applied to the plate. A very small difference in the quantity of material applied to duplicate spots can amount to a variation of 3 to 10γ depending on the concentration of the solution.

SUMMARY

Several solvents previously used in paper chromatography for the separation of sugars were evaluated for use with cellulose thin-layer plates. Several simple sugars which were difficult to separate by one-dimensional chromatography on either paper or silica gel can be separated on cellulose using a solvent of formic acid-methyl ethyl-ketone-tert. butanol-water.

Colorimetric procedures using anthrone and p-aminobenzoic acid were compared for their usefulness for the quantitative analysis of sugars separated by the above method. The anthrone procedure was recommended for its wider range of applicability.

The qualitative and quantitative procedures developed were then shown to be applicable to the analysis of plant extracts by identifying and quantitatively determining the free sugars extracted from various tissues of the cotton variety Acala 4-42.

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