CIRCULAR PAPER CHROMATOGRAPHY

Part VIII. Separation, Identification and Quantitative Estimation of Sugars and Oligosaccharides

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Summary

A technique for the separation and identification of sugars and oligosaccharides by circular paper chromatography is described. *n*-butanol-pyridine-water (60: 40: 30) and *n*-butanol-acetone-water (20: 70: 10) solvent mixtures are found to be effective for the separation of large number of sugars from a mixture. The R_f values for several sugars and some oligosaccharides in three solvent mixtures are given.

The technique of multiple development and its use in improving the resolution of sugars are described. The resolution of sugars can also be improved by reducing the rate of irrigation, which can be controlled by varying the size of the wick.

A method for the identification of sugars by treatment of the chromatogram with different reagents giving characteristic colour reactions with the sugars by multisector technique is given.

A quantitative method for the determination of reducing sugars separated on the paper is described. It is based on the reduction of triphenyl-tetrazolium chloride in alkaline solution to the insoluble red coloured formazan compound by the reducing sugars. The experimental conditions necessary to obtain accurate and reproducible results are indicated.

Large quantities of sugars can be separated on Whatman No. 3 Filter paper by multiple development technique, and a small-scale preparative method for sugars is possible by this technique.

A few examples of the application of this technique to problems in carbohydrate chemistry are given. The analysis of sugars and the study of carbohydrate metabolism are rendered simpler by this technique.

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The successful application of the circular paper chromatographic technique developed in this laboratory to the separation and identification of amino acids in protein hydrolysates and biological fluids (Giri, 1951; 1953;

Giri and Rao, 1952 *a*, *b*; Giri *et al.*, 1952, *c*, *d*, *e*; Airan and Master, 1953; Airan and Karat, 1953; Prior and Whitehead, 1953) and their quantitative estimation (Giri *et al.*, 1952, *f*; 1953, *b*, *d*); organic acids (Giri *et al.*, 1953, *a*; Airan *et al.*, 1953 *a*); purines (Giri *et al*, 1953, *c*); phosphoric esters (Ganguli, 1953), antibiotics (Brockmann and Gröne, 1953) and to inorganic analysis (Airan, 1952; Airan and Barnabas, 1953) suggested the general usefulness of this technique for carbohydrates.

Rao and Beri (1951) using Rutter's technique separated sugars from a mixture containing two to four sugars and determined the R_f values in moist phenol, butanol, S. collidine, methyl-ethyl ketone and *p*-cresol. They could not separate glucose and galactose by using these solvents. Krishnamurthy and Venkitasubramanyan (1952) employed the technique developed in this laboratory for the separation of sugars from fruits and reported the R_f values of ten sugars. Bersin and Muller (1952) using the drop method of Zimmermann and Nehring (1951) separated lactose, glucose, xylose and Rhamnose from a mixture of these sugars, in butanol-acetic acid-water (4:1:5) as solvent mixture and determined the R_f values of ten carbohydrate substances. Recently Schwertfeger (1953) showed that the resolution of substances (amino acids) by feeding with the 'wick' method was better than that obtained by the drop method of Zimmermann and Nehring (1951). Luderitz and Westphal (1952) applied Rutter's technique to the separation of rhamnose, xylose, glucose and galactose from a mixture using pyridine-butanol-water as solvent. The number of sugars separated on a single chromatogram by all the above authors was limited to four only. The object of the present investigation is to show that it is possible to achieve improved separation of large number of sugars from a mixture on a single chromatogram by the use of suitable solvents and improved techniques developed in this laboratory and to indicate the general applicability of this technique in carbohydrate research. A method for the quantitative determination of reducing sugars after separation on the paper is also described.

EXPERIMENTAL

The apparatus and general procedure for running the chromatograms were exactly the same as described in earlier publications (Giri and Rao, 1952, *a*, *b*).

Sugars

The sugars used in this investigation were obtained from Nutritional Biochemical Corporation, Ohio. The solutions for chromatography were prepared by dissolving 1 gram of each sugar in water and making up the

volume to 100 c.c. Isomaltose was prepared from the culture filtrate of the mold *Penicillium chrysogenum*-Q-176, grown on maltose containing medium, by fractionation on charcoal column (Giri, K. V., Saroja, K., Venkataraman, R. and Narasimha Rao, P. L., unpublished work). A sample of β-isomaltose-octa-acetate was kindly supplied by Dr. A. Thompson of the Ohio State University, U.S.A., from which isomaltose was prepared by deacetylation and used for confirming the identity of isomaltose prepared in this laboratory. Maltotriose and maltotetraose were obtained from Professor S. Peat of the University College of North Wales, Bangor. Gentiobiose was obtained from Dr. W. J. Whelan, University College of North Wales, Bangor.

Solvents

The solvent mixtures investigated in this laboratory for the resolution of the sugars are described below:

All the solvents used in the present investigation were distilled before use.

1. n-*Butanol-acetone-water* (20 : 70 : 10).—To 70 c.c. of distilled acetone were added 20 c.c. of *n*-butanol and 10 c.c. of water. This was found to be an excellent solvent mixture for the separation of sugars.

2. n-*butanol-acetic acid-water* (40 : 10 : 50).—This solvent mixture, which was recommended by Partridge (1947) for the separation of sugars, was not found to be as good as the above solvent for the separation of large number of sugars into distinct bands.

3. n-*Butanol-pyridine-water* (60 : 40 : 30).—The use of this solvent mixture in the proportions 3 : 1 : 1 was used by Hough *et al.* (1950) and reported that it gave reproducible results. By increasing the pyridine or water content of the mixture, the R_f values could be increased. The solvent mixture used in the present investigation was prepared by mixing 60 c.c. of *n*-butanol, 40 c.c. of pyridine and 30 c.c. of water. This solvent gave excellent and satisfactory resolution of sugars.

Reagents for detection and identification of sugars

The following reagents were used for the detection and identification of sugars and oligosaccharides separated on the chromatogram.

1. *Aniline-Diphenylamine-phosphate*.—The reagent was used by Buchan and Savage (1952) for the detection of sugars. They found that it is superior to other reagents for the location of sugars on the chromatogram.

The reagent was prepared by mixing the following solutions before use in the proportions stated below:—

(i) 4 per cent. solution of aniline in 95 per cent. alcohol (5 volumes);

(ii) 4 per cent. solution of diphenylamine in 95 per cent. alcohol (5 volumes);

(iii) Syrupy phosphoric acid (1 volume).

The reagent has the advantage of being effective for all reducing and non-reducing sugars and oligosaccharides and is therefore of general application to all sugars. It produces blue, green, yellow or brown coloured bands with sugars, the nature of the colour depending on the type of sugar.

2. *α-Naphthylamine-phosphoric acid.*—A 0·5 per cent. solution of α-naphthylamine in 95 per cent. alcohol was prepared. 10 c.c. of this reagent and 2 c.c. of syrupy phosphoric acid were mixed before use. This reagent is highly specific for ketoses, other sugars giving very faint or no colour with this reagent. The colour produced with this reagent was yellow for fructose, sorbose and all sugars and oligosaccharides containing fructose residue. The intensity of the colour of the bands was considerably improved when observed under ultra-violet light (Wood's filter).

3. *Aniline hydrogen phthalate* (*Partridge,* 1949).—The reagent was prepared by adding aniline (0·93 gm.) and phthalic acid (1·66 gm.) to water-saturated *n*-butanol (100 c.c.).

This reagent is very sensitive for aldopentoses and aldohexoses. The aldopentoses give bright red colour and the aldohexoses give brown colour.

4. *Triphenyltetrazolium chloride* (*T.T.C.*).—This reagent was prepared by mixing immediately before use equal volumes of a 2 per cent. solution of T.T.C. in water saturated butanol and 1 N NaOH in 95 per cent. alcohol. All reducing sugars give deep red colour with this reagent.

PROCEDURE

All the experiments described in this paper except those for the separation of large quantities of sugars were carried out on Whatman No. 1 circular filter-papers of 30– –35 cm. or 18·0 cm. diameter. The chromatograms were run without prior equilibration with the vapours of the solvent.

The volume of the experimental solution to be spotted depends on the size of the chromatogram and the concentration of the sugars in the solution. For large size chromatograms (35 cm. diam.) 70–80 μl. containing 700–800 μg. of each sugar was spotted at the centre of the paper. About

10 μl. (spot diam. approx. 5 mm.) was spotted at a time and each spot was dried at room temperature before another was superimposed on it. The volume of the solution spotted can be reduced considerably if more concentrated sugar solutions are used. When a number of different samples were run on the same paper (multisector circular chromatogram), the samples (5–10 μl. containing 50–100 μg. of each sugar) were spotted on the circumference of a circle (4 cm. diam.) drawn with a pencil at the centre of the paper. After drying the spots at room temperature, the paper was irrigated with the solvent mixture (*n*-butanol-pyridine-water (60 : 40 : 30). The time taken for the solvent boundary to reach the edge of the paper (35 cm. diam.) was about 10–12 hours. After irrigation, the paper was removed, and dried at room temperature. The irrigation was again repeated. This was found to be necessary for better resolution of the bands. After the paper was dried in air, the position of the sugars on the paper was rendered visible by treating the paper with a suitable reagent.

Application of the reagents.—The paper after drying was placed on a sheet of glass slightly larger than the paper itself. The reagent was applied to the paper with a camel hair brush. This procedure proved not only to be simple and elegant, but also permitted the identification of sugars by treatment of different sectors of the paper with different reagents without cutting the paper into sectors. Further, the chromatograms obtained by treating the paper with the reagents by the brushing technique were found to be better than those obtained by spraying technique, as the latter showed irregular patches on the paper, when aniline-diphenylamine-phosphoric acid reagent was used for spraying. The T.T.C. reagent was applied by means of cotton.

Immediately after application of the reagent, the paper was dried at 100–105° C. until all the bands appeared giving rise to well-defined bands of different colours with varying intensities depending on the type of sugar and the reagent used for identification. The chromatogram treated with the T.T.C. reagent was dried in humid atmosphere according to the procedure described later in the paper.

A typical chromatogram is illustrated in Fig. 1 which shows the separation of 10 sugars from a mixture. *n*-butanol-pyridine water (60 : 40 : 30) was used as the solvent mixture and aniline-diphenylamine-phosphoric acid as the reagent to reveal the position of the sugars.

R_f values of sugars.—In Table I are presented the R_f values of the sugars in different solvents. The sugar solutions (5 μl.) were spotted on the circumference of the circle drawn at the centre and after development the paper

was treated with aniline-diphenylamine-phosphoric acid reagent. As the R_f values vary with the experimental conditions they merely serve to indicate the position of the sugars on the chromatogram. The relative position of the sugars, however, remains unaltered in a particular solvent. The R_f values are generally higher than those reported by other workers using the conventional ascending and descending techniques.

TABLE I

R_f values of sugars in various solvents on Whatman No. 1 paper (30 cm. diam.)

Sugars	Acetone 70 Butanol 20 Water 10	Butanol 40 Acetic acid 10 Water 50	Butanol 60 Pyridine 40 Water 30
1. Maltotetraose	0·15	0·10	0·21
2. Maltotriose ..	0·25	0·17	0·29
3. Raffinose	0·27	0·16	0·25
4. Galacturonic acid ..	0·24–0·34 (two bands)	0·24–0·26 (two bands)	0·12–0·35 (two bands)
5. Melibiose	0·35	0·20	0·28
6. Melizitose	0·35	0·21	0·31
7. Lactose	0·36	0·21	0·31
8. Isomaltose ..	0·36	0·23	0·30
9. Gentiobiose ..	0·36	..	..
10. Cellobiose ..	0·37	0·24	0·35
11. Maltose	0·38	0·25	0·35
12. Sucrose	0·45	0·30	0·40
13. Galactose	0·49	0·35	0·43
14. Glucose	0·53	0·38	0·48
15. Sorbose	0·61	0·41	0·49
16. Fructose	0·62	0·44	0·49
17. Mannose	0·62	0·44	0·48
18. Arabinose	0·63	0·44	0·49
19. Fucose	0·64	0·52	0·54
20. Xylose	0·65	0·46	0·53
21. Ribose	0·68	0·50	0·59
22. Rhamnose ..	0·71	0·57	0·70
Average distance of solvent front from the centre of the filter paper	14 cm.	14 cm.	14 cm.

Multiple development technique.—The multiple development technique, first suggested by Jeanes *et al.* (1951) to improve the resolution of sugar mixtures, was applied successfully to the separation of amino acids by circular paper chromatographic technique (Giri, 1951; Giri and Rao, 1952, *a*). This technique was found to be very useful in improving the resolution of sugars by the circular technique. A typical multi-sector semi-circular chromatogram is shown in Fig. 2 which shows the improvement in the resolution of the sugars on repeated development. The mixture (about 10 μl.) containing 10 sugars was spotted at position marked (3) on the circumference of a circle (4 cm. diam.) drawn with a pencil at the centre of a circular filter-paper (35 cm. diam.). After drying the spot, it was developed with *n*-butanol-pyridine-water until the solvent reached very near the edge of the paper. It was taken out and dried. The mixture was again spotted at the position marked (2) and the development repeated until the solvent front reached the same position as before. The development was again repeated for the third time after the mixture was spotted at position (1). Thus the sector (1) was developed once, while the other sectors 2 and 3 were developed twice and three times respectively. The paper was dried and after treatment of the paper with the reagent aniline-diphenylamine-phosphoric acid, it was finally dried at 100–105° C. until all the bands of the sugars appeared on the paper.

It can be seen from Fig. 2 that the sector which was developed once, showed diffused bands, while the second and third sectors indicated considerable improvement in the resolution of the sugars. It is, therefore, desirable that the development should be repeated again for the second time to achieve better resolution. This is absolutely necessary when a large number of sugars are present in a mixture, and have to be separated into clear and well-defined bands.

Influence of size of the wick on the rate of development and resolution of sugars.—The rate of irrigation could be controlled by varying the size of the wick. By reducing its size the rate of irrigation could be decreased resulting in improved separation and sharpness of the bands. Increase in the rate of irrigation resulted in poor resolution with diffused bands. It is, therefore, necessary to choose a proper wick to suit the size of the paper for better resolution of the sugars. This is clearly illustrated in Fig. 3. Even if the wick is not of proper size, multiple development will always improve considerably the resolution of the sugars. Similar observations were made with amino acids (Giri, K. V. and Tara Rao, unpublished work).

Identification of sugars by colour reagents.—As the relative rates of movement of sugars are constant in a particular solvent, the band relating

to any one sugar can be identified by its position relative to known bands of sugars, separated on the same paper after spraying with a suitable reagent to reveal the position of the sugars. This can be easily carried out by the multi-sector technique (Giri and Rao, 1952, *a*, *b*). The identification of the sugars can be further confirmed by carrying out tests with reagents which react with the sugars to produce characteristic colour reactions.

The procedure for running the multi-sector chromatograms for carrying out the specific colour reactions, consisted in spotting the solution containing the mixture of sugars at the centre of the paper. After development and drying, the paper was divided into sectors with a pencil. The reagents were applied to each sector with a camel hair brush or cotton as described before and the paper was dried at 100–105° C.

Fig. 4 is the multi-sector semi-circular chromatogram showing the method of identification of the sugars by this technique.

Table II shows the colour rections given by the sugars with the respective reagents.

The colours obtained with the different reagents varied with the type of sugar, thus permitting their characterisation. Rhamnose reacted slowly with the reagent aniline-diphenylamine-phosphoric acid. Some variation of the colours of the bands on the chromatograms treated with aniline-diphenylamine reagent was observed after keeping it for some time. The yellow colour of the rhamnose band changed slowly to green. The change in the colour was also observed in the case of other sugars. The background also assumed light blue colour after about 48 hours. Temperature and duration of heating influenced the tone and colour intensity of the bands. The examination of the chromatograms after treatment with the reagents, under ultraviolet light (Woods filter) showed differentiation of the colours of the bands and the intensity of the colours was very much increased. This was strikingly shown in the case of ketoses and all sugars and oligosaccharides containing fructose residue treated with α-naphthylamine-phosphoric acid which is a specific reagent for these sugars. For the identification of pentoses aniline-hydrogen-phthalate (Partridge, 1949) is very useful. T.T.C. is a general reagent for all reducing sugars. It was of special interest that the 1 : 4 and 1 : 6 glycosydic linkages of the sugars influenced the colour reaction. Thus maltose (1 : 4 linkage) and iso-maltose (1 : 6 linkage) could be readily differentiated by their characteristic colours, the latter giving brownish yellow and the former blue colour when treated with aniline-diphenylamine-phosphoric acid reagent.

TABLE II

Colour reagents used in the identification of sugars and oligosaccharides

Sl. No.	Compound	Colour of the band			
		Aniline-Diphenylamine phosphoric acid	Aniline hydrogen-phthalate	α-Naphthyl amine-phosphoric acid	Triphenyl-tetra-zolium chloride
1	Maltotetraose	Blue	Brown	—	Deep red
2	Maltotriose..	Blue	Brown	—	Deep red
3	Raffinose ..	Dark yellow	—	Yellow	—
4	Malezitose ..	Dark Yellow	—	Yellow	—
5	Lactose ..	Blusish green	Light brownish yellow	—	Deep red
6	Isomaltose ..	Yellowish brown	Brown	—	Deep red
7	Gentiobiose	Yellowish brown			Deep red
8	Melibiose ..	Yellowish brown	Brown	—	Deep red
9	Maltose ..	Blue	Light brown	—	Deep red
10	Cellobiose ..	Blue	Light brown	—	Deep red
11	Sucrose ..	Dark yellow	—	Yellow	—
12	Galactose ..	Dark yellow	Brown	—	Deep red
13	Glucose ..	Dark yellow	Brown	—	Deep red
14	Sorbose ..	Light dark yellow	Light brown	Yellow	Deep red
15	Fructose ..	Light dark yellow	Light brown	Yellow	Deep red
16	Mannose ..	Bluish green	Brown	—	Deep red
17	Arabinose ..	Greenish-yellow	Red	Very light grey	Deep red
18	Xylose ..	Greenish yellow	Red	—	Deep red
19	Ribose ..	Greenish yellow	Red	—	Deep red
20	Fucose ..	Yellow	Brown .	—	Deep red
21	Rhamnose ..	Yellow	Yellowish brown	—	Deep red
22	Glucosamine	Green	Reddish brown	—	Deep red
23	Galacturonic acid ..	Brown	Reddish brown	—	Deep red
24	Gluconic acid lactone	—	—	—	—
25	Ascorbic acid	—	—	—	Deep red

– Indicates no colour.

Quantitative estimation of reducing sugars separated on the chromatogram.—The method adopted for the quantitative estimation of the reducing sugars separated on the chromatogram consisted in treating the paper after development with triphenyl-tetrazolium chloride (T.T.C.) reagent and determining the intensity of the red colour of the bands after extraction with alcohol. The reagent T.T.C. dissolves in water to form a colourless solution. In presence of reducing sugars in alkaline medium, it is converted to triphenyl formazan, which is an intensely red coloured water insoluble compound. The quantity of formazan formed is proportional to the amount of reducing sugar. This property of T.T.C. was utilised by Mattson and Jensen (1950) for the colorimetric determination of reducing sugars and applied to the analysis of lactose in milk and glucose and fructose in honey. The reagent was subsequently used by Trevelyan *et al.* (1950) for the detection of reducing sugars on paper chromatograms. Wallenfels (1950) indicated its use in the quantitative estimation of sugars on paper. He used a freshly prepared solution of 2 per cent. aqueous T.T.C. and 1 *N*-sodium hydroxide mixed in equal proportions as spraying reagent. The colour was extracted with pyridine containing 10 per cent. HCl and estimated colorimetrically. The reagent was also used by Luderitz and Westphal (1952) for the estimation of carbohydrate constituents of pyrogen polysaccharides of *B. coli.*

A detailed investigation on the use of T.T.C. for the quantitative determination of reducing sugars separated on circular paper chromatograms was undertaken. It was found that by heating the paper at high temperatures without proper control of the humidity, background colour developed, which interfered with the estimations. After a number of trials a procedure was standardised for the control of temperature and moist atmosphere favourable for maximum colour development without the formation of background colour. Furthermore, the use of aqueous solutions of the reagent caused spreading of the bands of sugars separated on the paper with consequent poor definition. This difficulty was eliminated by dissolving the reagent in water-saturated butanol and using alcoholic solution of potassium hydroxide.

Reagents: 1. Potassium hydroxide solution 1·0 N. in 95 per cent. alcohol.

2. Triphenyl tetrazolium chloride (B.D.H.). 2 gm. of this reagent was dissolved in 100 c.c. of water saturated butanol. The reagent is sensitive to light. It should therefore, be stored in a brown bottle and kept in a refrigerator.

The reagents were mixed in equal proportions immediately before use.

Procedure.—Standard solutions containing varying concentrations of each sugar were spotted by means of a 5 μl micropipette, on the circumference of a circle (3 cm. diam.) drawn at the centre of a circular filter-paper (18 cm. diam.). The spots were allowed to dry in air and the chromatogram was run as usual with *n*-butanol-acetone-water as the solvent mixture. Double development was carried out in each case, and the solvent front was allowed to move a distance of about 8 cm. from the centre. After the development was completed, the paper was dried at room temperature and treated with the T.T.C. reagent by placing the paper on a glass plate and applying the reagent to the paper with a piece of cotton. This procedure was found to be simple and economical and clear chromatograms were obtained. Immediately after application of the reagent, the paper was kept over a petridish (6 in. diam.) half filled with water, and covered with another petridish of the same diameter. The whole set-up was placed in a uniformly heated water oven at 40–50° C. The temperature of the oven was allowed to rise slowly so as to attain a temperature of 75-80° C. in about 30 minutes. The paper was allowed to remain at this temperature for 25–30 minutes, when intensely coloured red bands appeared on the paper. It was then removed, washed with water and dried at room temperature. After this treatment, red coloured bands appeared on the chromatogram, against little or no background colour. The bands were cut out and the red colour was extracted with 12·5 c.c. of 95 per cent. alcohol. The colour was completely extracted in 2 to 3 hours. The formazan formed was completely soluble in 95 per cent. alcohol to give a clear red coloured solution. The intensity of the colour was measured in a Klett-Summerson photoelectric colorimeter using Filter No. 54. Upon plotting the colorimeter reading against sugar concentration straight line graphs were obtained over the range 5–100 μg. of the sugars investigated. It was also observed that there was no significant differences in the colour intensities when other solvents—butanol-acetic acid-water and butanol-pyridine-water—were used. The colour of the alcoholic extract was found to be stable for more than 24 hours when kept in a refrigerator.

The intensity of the colour given by an equivalent amount of different sugars differed with the type of sugar, the ketose-fructose and sorbose giving higher values than other sugars, and disaccharides in general gave values lower than the corresponding monosaccharides.

The quantitative analysis of a mixture of sugars can be carried out by spotting the solution to be analysed on the circumference of a circle drawn at the centre along with the standard solutions of the sugars spotted on either side of the test sample. By comparing the intensity of the colour of the test sample with that of known concentration of the sugar, the amount of sugar

present in the test sample can be determined. This procedure should always be preferred to the one in which the values are obtained from the calibration curves, since the intensity of the colour of the bands may vary slightly with experimental conditions.

The quantitative analysis of the sugars separated on the chromatogram can also be carried out either by the method described by Hirst *et al.* (1947) or by the procedure described by Hawthorne (1947).

Application to carbohydrate analysis.—The method can be applied to the analysis of carbohydrate materials such as malt syrup, liquid glucose, honey and fruit juices and concentrates and other carbohydrate materials. It can be applied to the identification of sugars in urine and blood. The identification of these substances in urine and blood is very difficult when traces of these substances are present or when two or more sugars or reducing substances occur in urine. The separation and identification of these substances by this technique can be readily achieved in a clinical laboratory.

The technique was applied in this laboratory to the study of the mechanism of amylolysis by characterising the sugars and oligosaccharides formed during the hydrolysis of amylose and starch by different types of amylases (Giri *et al.*, 1953 *e*). The enzymic synthesis of oligosaccharides from maltose and sucrose by penicillium chrysogenum Q-176 was studied by this technique (Giri *et al.*, 1953 *f*). It can be applied to a wide variety of problems in carbohydrate chemistry.

DISCUSSION

The R_f values given in Table I show that the values are in general high in butanol-acetone-water and butanol-pyridine-water solvent mixtures compared to the values in butanol-acetic acid-water solvent. The wide distribution of the R_f values in the first two solvent mixtures is favourable to the separation of large number of sugars in a mixture. Among the three solvents *n*-butanol-pyridine-water was found to be the most efficient solvent for the separation of sugars. *n*-butanol-acetone-water was also quite satisfactory after second development.

The R_f values indicate the relative position of the sugars with respect to each other and also give general indication of the degree of separation obtainable with each solvent mixture. To obtain constant R_f values it is necessary to control all the variables affecting the values. In routine application it is not possible to control these variables. As the relative rates of movement of sugars in any particular solvent are constant without any control of the variables, the sugars can be identified by its position relative

to known bands of sugars. The identity can further be confirmed by the characteristic colour tests with specific reagents. It is always preferable to spot known sugars alongside " unknowns " on the same paper and compare the position of the bands and the colours obtained with the various reagents. In some cases further confirmation is necessary by separating the unknown sugar by chromatography on a column of cellulose (Hough *et al.*, 1949) or charcoal celite (Whistler and Durso, 1950) and determining the physical constants and preparing the characteristic derivatives.

The overlapping sugars can be identified by carrying out specific colour reactions by the multi-sector technique (Fig. 4). For example, if fructose is present in the mixture containing arabinose they overlap and appear as a single band on the chromatogram treated with aniline-diphenylamine reagent. The presence of fructose can be confirmed by applying the α-naphthylamine reagent to one of the sectors and the other reagents to the adjoining sectors. The formation of a yellow band corresponding to arabinose band on the sector indicates the presence of fructose in the mixture.

For the separation of large number of sugars multiple development was always preferred to single development, as the resolution of sugars was considerably improved after second development (Fig. 2). The rate of irrigation also influenced the resolution of sugars, the slower the rate of irrigation, the greater was the improvement in the resolution of sugars (Fig. 3).

The quantitative method described in this paper was found to be simple, convenient and accurate to within $\pm$ 5 per cent. In order to obtain consistent and accurate results with T.T.C., it was found necessary to spot on the same paper standard solutions of the sugars on either side of the test sample, and determine the colour intensities of the bands separated on the paper.

Some preliminary experiments were carried out with the object of finding out the possibility of separating large quantities of sugars by this techniuqe. Whatman No. 1 paper could not be used without a risk of " overloading " the paper. The separation of the bands was not distinct and identification was rather difficult. The use of thick paper Whatman No. 3 was found to be useful for the separation of large quantities of sugars on 35 cm. diameter paper by multiple development technique. Nearly 1 c.c. of sugar solution containing 10 mg. of each of ten sugars was spotted and separated into ten distinct bands after four developments. It is, therefore, possible to separate not only micro-quantities of sugars but also macro-quantities by using large and thick papers and employing the multiple development

technique, thus facilitating the characterisation of sugars after elution by the standard chemical and physical methods. The technique should be of wide application as a micro- as well as a macro-method of investigation in carbohydrate chemistry.

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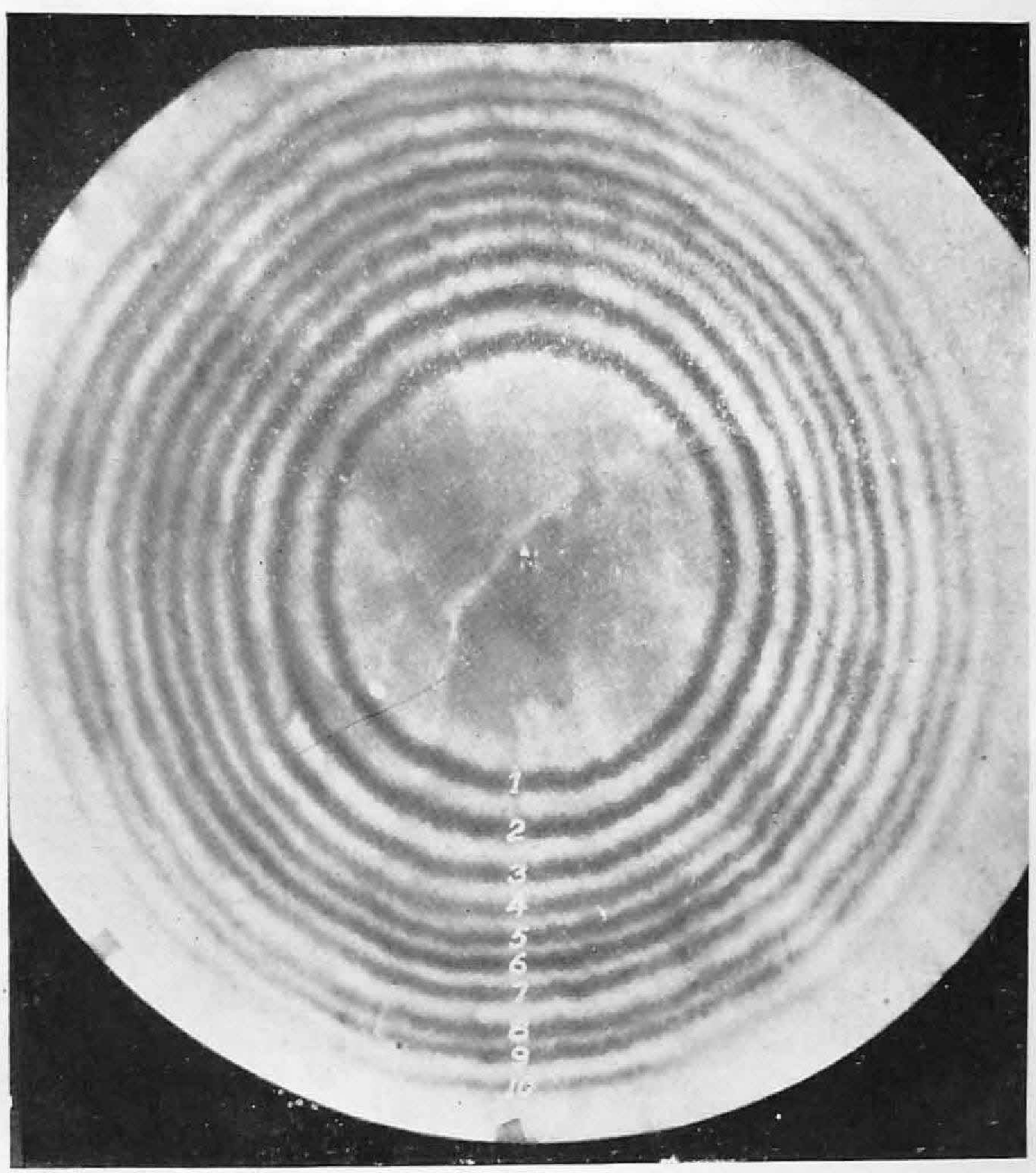


FIG. 1. Circular paper chromatogram of Sugars. Solvent, *n*-Butanol-pyridine-water (60: 40: 30). Whatman No. 1 paper, 35 cm. diameter; Double development 80 μ of solution containing 0·8 mg. of each sugar spotted at centre. Raffinose; 2. Lactose; 3. Maltose; 4. Sucrose; 5. Galactose; 6. Glucose; 7. Arabinose; 8. Xylose; 9. Ribose; 10. Rhamnose.

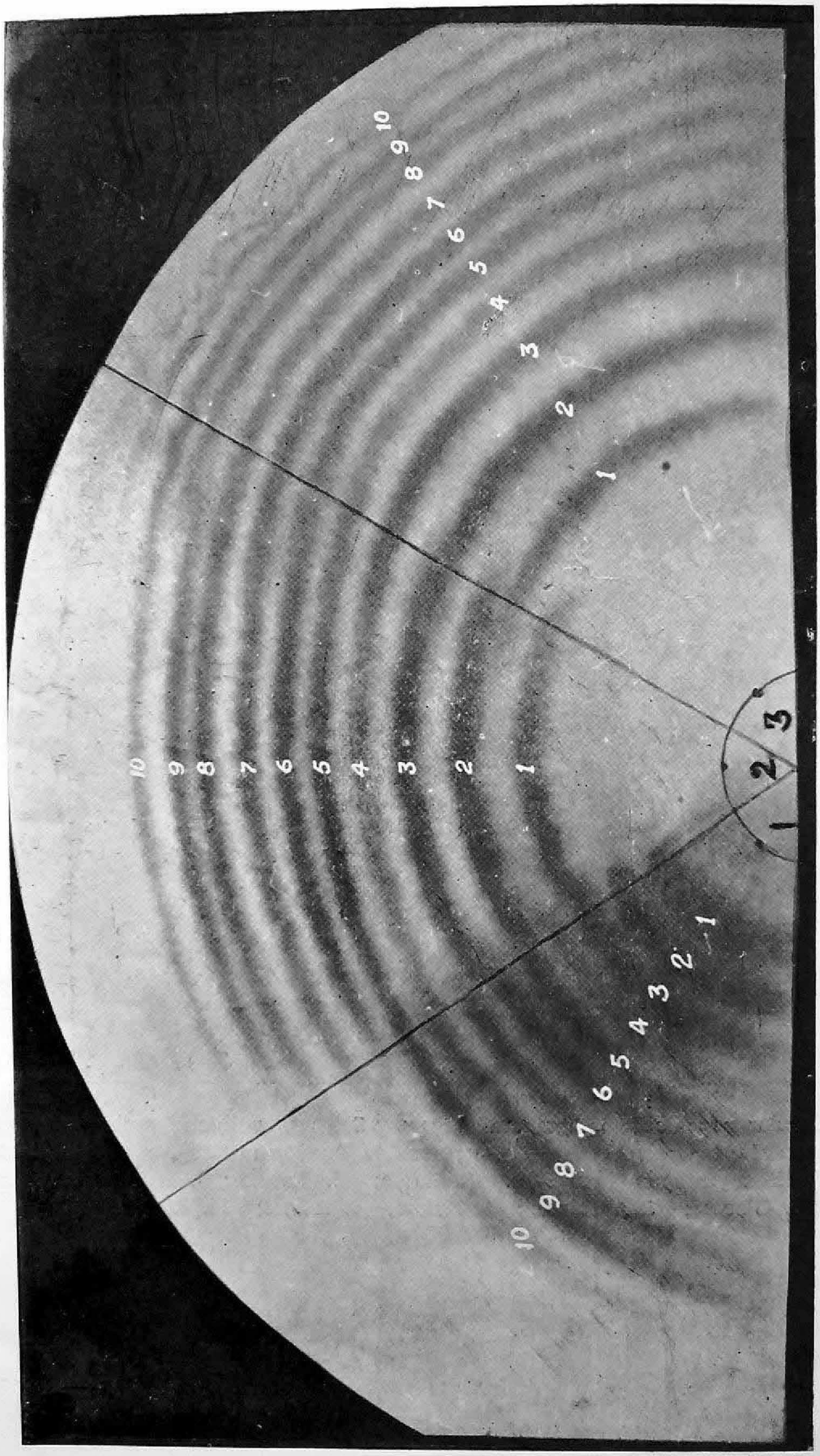
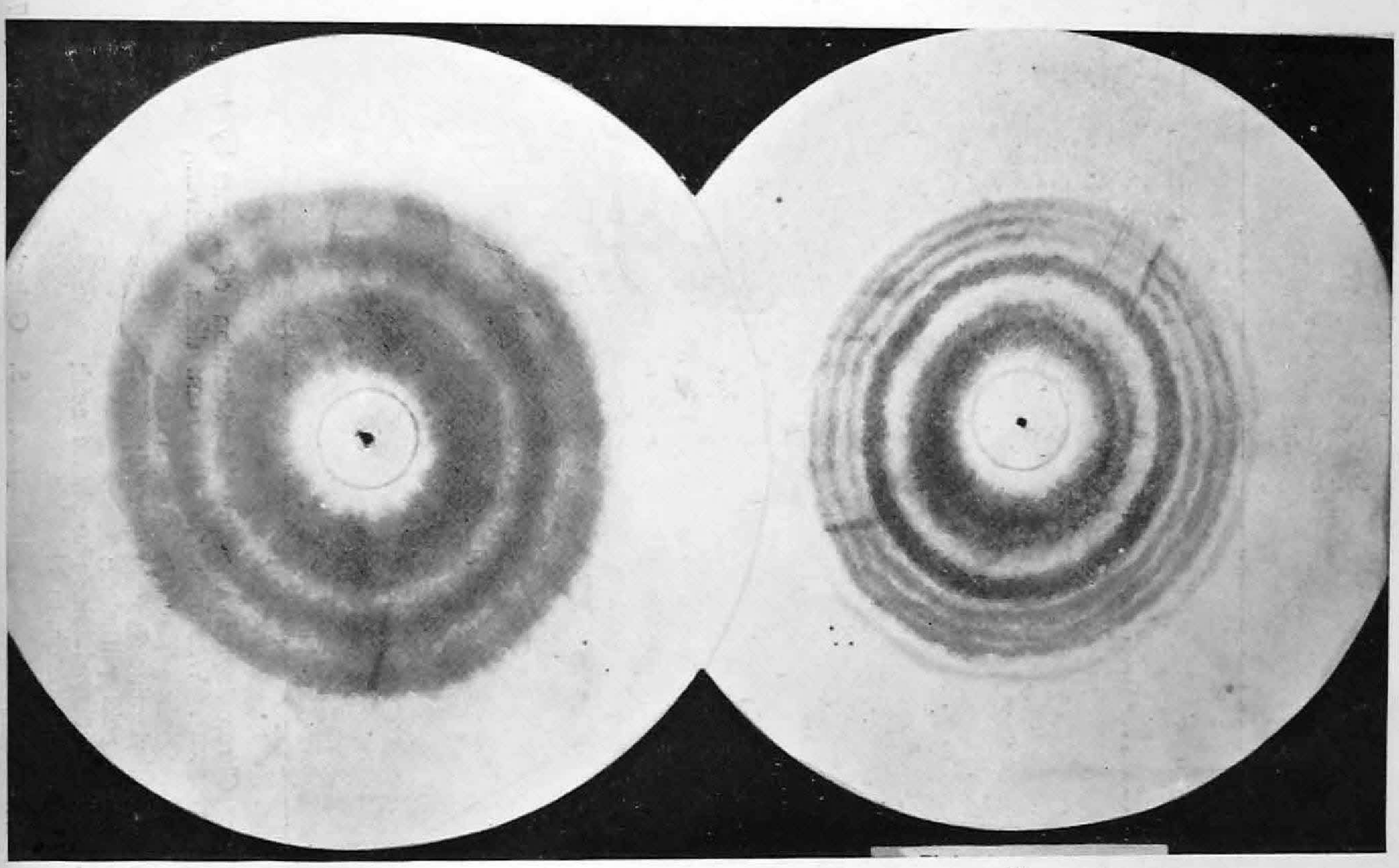


FIG. 2. Multi-sector chromatogram showing improvement in the resolution of sugars by Multiple Development Technique. 1-Developed once; 2-Developed twice, 3-Developed thrice; Solvent:, *n*-Butanol-pyridine-water (60: 40: 30). Whatman No. 1 paper (35 cm. diameter). 1. Raffinose; 2. Lactose; 3. Maltose:, 4. Sucrose; 5. Galactose; 6. Glucose; 7. Arabinose; 8. Xylose; 9. Ribose; 10. Rhamnose.



A B

FIG. 3. Chromatograms showing improvement in the resolution of sugars by controlling the rate of irrigation. A. Wick size—4·5×2·7 cm.; Time of irrigation, 2 hours. B. Wick size, 4·5×0·5 cm.; Time of irrigation, 7 hours. Whatman No. 1 (18 cm. diameter). Solvent, *n*-Butanol-pyridine-water (60: 40: 30); single development. 25 μl. of the sugar solution containing the same number of sugars as in Fig. 1 spotted at the centre.

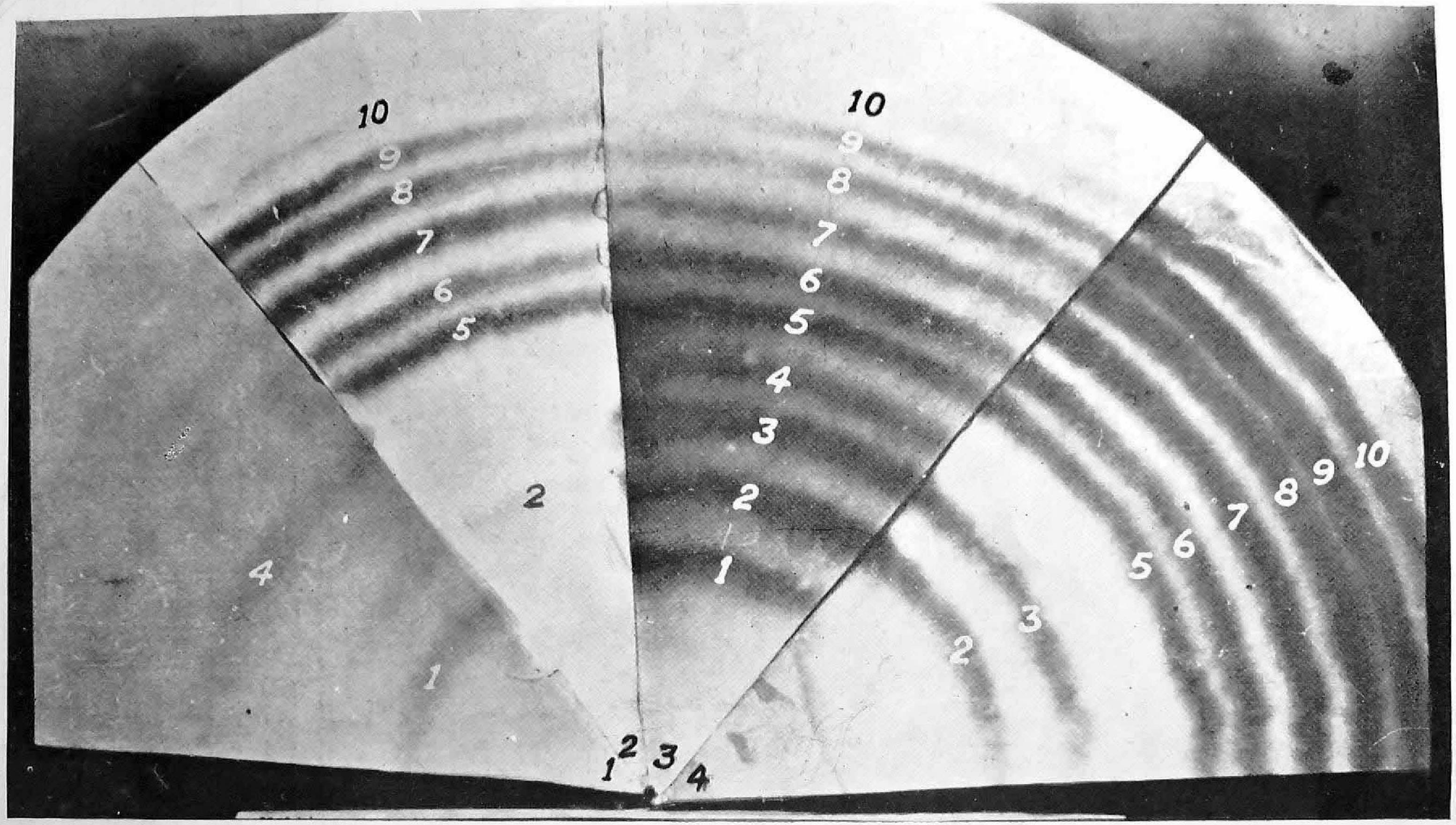


FIG. 4. Multisector Chromatogram showing identification of sugars by treatment of the sectors with different reagents. Whatman No. 1, 35 cm. diam. Solvent, Butanol-pyridine-water (60: 40: 30).

Sector 1. α-Napthylamine-phosphoric acid;
Sector 2. Aniline acid phthalate;
Sector 3. Aniline-diphenylamine-phosphoric acid;
Sector 4. Triphenyl-tetrazolium chloride.

1. Raffinose; 2. Lactose; 3. Maltose; 4. Sucrose; 5. Galactose; 6. Glucose; 7. Arabinose; 8. Xylose; 9. Ribose; 10. Rhamnose.

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