A RAPID QUANTITATIVE METHOD FOR ROUTINE DETERMINATION OF MONOSACCHARIDES AND OLIGOSACCHARIDES FROM PLANTS BY PAPER CHROMATOGRAPHY*

BOCK G. CHAN AND JOHN C. CAIN
New York Agricultural Experiment Station, Cornell University, Geneva, N.Y. (U.S.A.)
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INTRODUCTION

Methods of quantitative determination of sugars by paper chromatography can be divided into two groups. The first group quantitatively determines the colored compound that is formed on a chromatogram upon oxidation of a sugar by an oxidizing agent. The colored compound is either extracted and determined spectrophotometrically or determined by a transmittance densitometer. Such a compound is triphenyltetrazolium chloride which is reduced to a formazon compound which is extracted and determined by a spectrophotometer (WALLENFELS[1], MATTSON AND JENSEN[2] and TRAVELYAN *et al.*[3]). Another such compound is ammoniacal silver nitrate which upon reduction by a reducing sugar to silver black is measured with a transmittance densitometer (McFARREN *et al.*[4]) or a radiometer (BEER[5]).

The second group of methods extracts or elutes the sugar from a cut-out spot on a paper chromatogram in which the sugar was first located by a guide strip technique. Then the sugar is determined by a standard micro-colorimetric method (WHISTLER[6] and HOUGH AND JONES[7]). The latter method is preferred because sugars are easily and quantitatively extracted or eluted off the chromatogram with water and the color development which is formed by the reaction of a sugar and a reagent is more easily controlled in a test tube than that on the paper chromatogram. Extraction is done either by an extractor (DUFF AND EASTWOOD[8]) or simply by immersing the spot on the paper in water (WHISTLER AND HICKSON[9] and DUBOIS *et al.*[10]). SHALLENBERGER AND MOORES[11] simultaneously extracted and determined sugars separated on Whatman No. 1 paper. After paper blank correction, standard deviation was found to be less than 5 μg in the range of 10–200 μg of sugar. DIMLER *et al.*[12] modified the elution method of DENT[13] for sugar analysis and recommended that elution should be carried out at room temperature and the rate of elution should be carefully controlled to avoid channeling which causes incomplete elution.

This paper describes a rapid quantitative procedure with high reproducibility suitable for the large number of determinations involved in routine analysis for sugars in plants. The solvent which was developed gave good separation of sucrose, glucose and fructose, all of which are commonly found in plants. The sugars from

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the paper chromatogram were eluted by means of a micro-elution tube directly into the test tube used in a standard micro-colorimeter method.

MATERIAL AND METHODS

Extraction and purification of samples

Samples of 0.25 to 1 g of freeze-dried peach (*Prunus persica* var. Halehaven) endosperm were extracted with 50 ml of 70% ethanol or by the water–methanol–chloroform extracting method of BIELINSKI AND YOUNG[14]. The 70% alcoholic extract was partially purified by dilution with water and extraction with chloroform in the following tertiary solvent system: alcoholic extract, chloroform and water (6:3:2, v/v). The chloroform phase containing the fat-soluble materials was discarded. The aqueous phase was further purified by passing through a column of Dowex 50 resin in H^+ form and then a column of Amberlite IR 45 resin in OH^- form. Dowex 50 resin removed the cations, and Amberlite IR 45 resin removed the anions. The neutral filtrate and washings were combined and dried over concentrated sulfuric acid *in vacuo* in a desiccator. This residue was dissolved in a volume of 10% ethanol to make about 10 μg per λ, preparatory to colorimetric determinations of sugars and chromatography.

Determination of sugar concentration

The Somogyi copper reagent (SOMOGYI[15]) was prepared and stored in 2 parts (BELL[16]). A: 28 g anhydrous disodium phosphate and 40 g Rochelle salt were dissolved in 700 ml distilled water. 4 g sodium hydroxide were dissolved in 100 ml distilled water and added with mixing. 180 g anhydrous sodium sulfate were added and the whole was made to 900 ml. B: 8 g cupric sulfate (crystalline) were dissolved in 100 ml distilled water with a drop of sulfuric acid. Nine parts of reagent A and one part of reagent B were mixed just prior to use.

Arsenomolybdate reagent (NELSON[17]) was prepared as follows: 25 g ammonium molybdate were dissolved in 450 ml distilled water and 21 ml concentrated sulfuric acid were added slowly. Then 3 g dibasic sodium orthoarsenate pentahydrate in 25 ml distilled water were added with mixing. The solution was aged at 37° for 48 h before use and stored in a dark brown bottle.

One percent (v/v) invertase solution was prepared from concentrated invertase solution (Nutritional Biochemical Company). Two drops of the 1% invertase solution were added to each test tube containing sucrose and oligosaccharides with sucrose type of linkage (melizitose, raffinose, gentianose and stachyose). One to two hours at 20–25° should elapse before determination of sugar concentration.

The concentration of total reducing sugar and total sugar by hydrolysis with invertase was determined on the partially purified extracts. A specific volume of the partially purified sugar extract giving about 100 μg of sugar in 1 ml distilled water was mixed with 1 ml of the mixed Somogyi copper reagent. The mixed solution was heated in a water bath at 100° for 20 min, then cooled immediately in a water bath at room temperature. The 1 ml of arsenomolybdate reagent was added and mixed vigorously. After 5 min the solution was diluted to 25 ml final volume with distilled water and mixed, then measured at 630 mμ with a Bausch and Lomb Spectronic 20. The concentration of sugar was calculated from a standard glucose

curve established for each batch of reagents. 2 ml of the reagents instead of 1 ml was used in the sugar determination on eluates from paper chromatograms because the volumes of eluates were approximately 2 ml.

Chromatography

Four spots of each sample of sugar solution were placed 4 cm apart on Whatman No. 3MM paper with a Misco spotting pipette. The amount of sugar per spot should give a concentration of 10–150 μg for each sugar determined. When multiple spotting was found necessary to give this concentration, the spot was dried in an air stream of 60° before respotting. A standard sugar mixture consisting of raffinose, sucrose, glucose, fructose, arabinose and xylose was spotted on the edge of each chromatogram as references. A solvent which gave good separation of sucrose, glucose and fructose was water-saturated *n*-butanol, 95 % ethanol and trichloroethylene (6:2:2, v/v). Chromatograms were developed descendingly for 32 h at 32° without equilibration. Longer developing time was needed to separate the higher oligosaccharides.

Development temperature of 32° was chosen because it improved definition, separated in less time (particularly with the slowly moving oligosaccharides), permitted handling of a larger quantity of sugars, and it was easy to maintain (CHAN[18]).

The chromatograms were dried at room temperature. The chromatogram was then cut into strips. One strip of the sample (the guide strip) and the reference strip

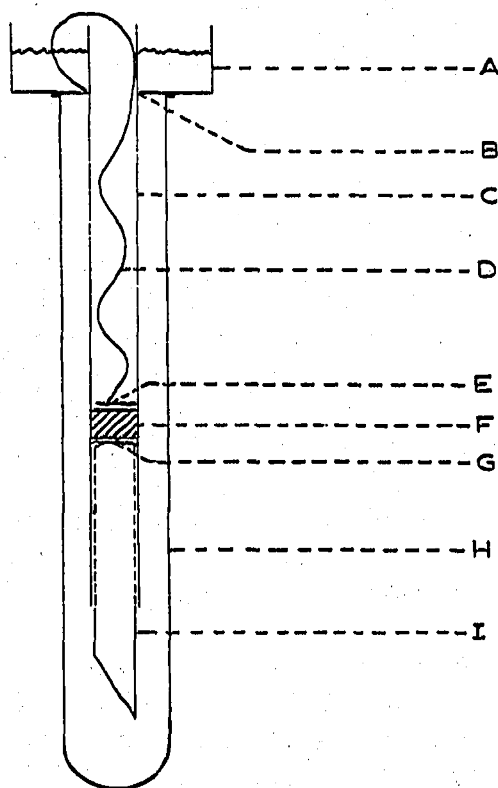

Fig. 1. Specifications for the assembly of the micro-elution tube. (A) a 1 cm in height × 3 cm in diameter plastic cap holding 3 ml water; (B) seal with paraffin; (C) 10 cm of a glass tube with 0.5 cm bore; (D) a strand from a glass wool wick 10 cm long; (E) loose cellulose powder; (F) 0.4 cm filter paper plug; (G) small plug of glass wool; (H) 15–150 mm test tube; (I) a rolled-up "cut-out square" of a paper chromatogram.

of the standard sugars were sprayed with the following improved benzidine reagent (CHAN AND CAIN[19]): A mixture of 10 ml of 5 % benzidine in glacial acetic acid plus 10 ml of 40 % aqueous trichloroacetic acid was diluted to 100 ml with 95 % ethanol. Aldoses were revealed as brown spots after heating at 70° for 10–15 min, then ketoses were revealed as yellow spots after additional spraying with 1.0 % HCl in 95 % ethanol and heating again at 70° for 10–15 min. These colored spots were marked with aid of a black light lamp. The chromatogram was reassembled. The sites of sugars on the three unsprayed strips were located by reference to the guide strip with a centimeter allowance on either end of the sugar spots.

The squares containing the sugars were cut out from the unsprayed strips of paper chromatogram and eluted by distilled water with a micro-elution tube. The specifications for the micro-elution tube are shown in Fig. 1.

The "cut-out square" of paper was rolled up diagonally with forceps. It was inserted half way into the micro-elution tube so that it touched the filter paper plug, which was adjustable. The pointed tip of the paper was squeezed, and the micro-elution tube was placed in a graduated test tube in a slightly tilted rack so that the tip of the paper was in contact with the test tube wall. This facilitated the run-off of the eluate. 3 ml distilled water were added to the plastic cup. The rate of capillary siphoning by the glass wool wick should be approximately equal to that of the paper so that little or no water accumulates above the filter paper plug. This held back excess water if siphoning was too rapid. The whole set-up was enclosed in a humidified box while elution proceeded overnight. To those test tubes collecting eluates containing sucrose, raffinose, or stachyose, two drops of 1 % invertase were added before elution. This allowed hydrolysis of these sugars as they were eluted into the test tube.

RESULTS

A solvent for paper chromatography was developed to give a good separation of sucrose, glucose and fructose. After a series of studies on various proportions of water-saturated *n*-butanol, 95 % ethanol and trichloroethylene, 6:2:2, v/v was found to be a good combination. Table I shows the comparison of the R_G values (reference to glucose) of some sugars by this solvent with several solvents studied by PARTRIDGE AND WESTALL[20]. Its R_G values compared favorably with the slowly developing *n*-butanol–NH_3 (1 % w/v) and better than the *n*-butanol–acetic acid–water (4:1:5, v/v).

Complete elution in the range of 47–142 μg glucose from 15 cm² Whatman No. 3MM paper was accomplished with 2.4 ml distilled water (Table II). Percentage of recovery varied from 101 to 102 %. The standard deviation of the mean was less than 1.1 μg glucose in the 47–142 μg range tested. 100 μg raffinose was eluted completely as shown by testing the paper with improved benzidine reagent afterward.

Sucrose, glucose, fructose and ribose were used to test the percentage of recovery and the reproducibility of the procedure. Table III shows a 91 to 99 % recovery with a standard deviation of less than 2 μg and with less than 3 % error.

The endosperm of peach, *Prunus persica* var. Halehaven, was extracted with 70 % ethanol and prepared for paper chromatography as described. Total reducing sugar and total sugar were determined on the partially purified extract. The total sugar was determined after hydrolysis by 1 % invertase. The difference between

TABLE I

A COMPARISON OF THE R_G VALUES OF SOME SUGARS IN WATER-SATURATED *n*-BUTANOL–TRICHLOROETHYLENE–95% ETHANOL (6:2:2, v/v) WITH SEVERAL SOLVENTS[20]

Sugar	*R_G value*	*R_G value calculated from Partridge and Westall*[20]		
	Water-saturated n-butanol–TCE–95% ethanol (6:2:2, v/v) at 22° for 36 h	*Phenol–HCN–NH_3 (1% w/v)*	*n-Butanol–HAc–water (4:1:5, v/v)*	*n-Butanol–NH_3 (1% w/v)*
Raffinose	0.07	0.69	0.28	—
Melibiose	0.16	—	—	—
Cellobiose	0.19	—	—	—
Sucrose	0.41	1.00	0.78	—
Galactose	0.76	1.13	0.89	0.86
Glucose	1.00	1.00	1.00	1.00
Fructose	1.42	1.31	1.28	1.43
Mannose	1.28	1.15	1.11	1.43
Arabinose	1.39	1.39	1.17	1.43
Xylose	1.83	1.13	1.56	1.79
Ribose	2.09	1.51	1.72	2.57

TABLE II

COMPARISON OF RECOVERY OF GLUCOSE BY ELUTION WITH DIRECT DETERMINATION BY A STANDARD COLORIMETRIC METHOD

Glucose (μg)		*Recovery (%)*	*Standard deviation (μg)*
Spotted	*Found**		
46.3	47.1	101.5	0.163
92.5	94.0	101.6	0.349
139.0	142.0	102.1	1.020
92.5**	—	—	1.455

* Mean of nine determinations.
** Mean of twelve determinations on 1 ml sugar solution.

TABLE III

RECOVERY OF SOME SUGARS FROM PAPER CHROMATOGRAMS

Sugar	*Quantity (μg)**		*Recovery (%)*	*Standard deviations (μg)*	*Errors (%)*
	Spotted	*Found*			
Sucrose	78.0	70.8	90.8	1.88	2.66
Glucose	71.0	70.5	99.3	2.01	2.85
Fructose	62.0	60.1	96.9	1.17	1.96
Ribose	92.1	90.5	98.3	1.49	1.62

* Mean of nine determinations.

TABLE IV

COMPARISON OF SUGAR DETERMINATIONS ON PEACH ENDOSPERM BEFORE AND AFTER CHROMATOGRAPHY
(mg glucose per g dried endosperm)

	Unidentified sugar	*Sucrose*	*Glucose*	*Fructose*	*Total reducing sugar*	*Total sugar*
Before chromatography	—	(29.2)*	—	—	100.8	130.0
After chromatography	4.20	26.6	46.3	38.1	(88.6)*	(115.1)*
Standard deviations (μg)**	0.114	0.174	0.028	0.199	0.220	0.078

* Values in parentheses are calculated.
** Standard deviation of nine determinations.

total sugar and total reducing sugar represented non-reducing sugar, the sucrose fraction. 91 % of this sucrose fraction was recovered as sucrose after chromatography (Table IV). Sucrose was further identified by invertase hydrolysis on the chromatogram as follows. The chromatogram was sprayed with 1 % invertase on the area containing sucrose and kept in a humidified chamber for several hours. It was developed at right angles to the first development with the solvent. When dry it was sprayed with benzidine reagent. The monomers of sucrose were identified as glucose and fructose by comparison with standards. Other sugars found in appreciable quantities were fructose, glucose and an unidentified reducing sugar; the percentage of errors was from less than 1 % for glucose to 2.7 % for the unidentified sugar.

DISCUSSION

The described method provided a simple procedure for quantitative sugar analysis of plants by paper chromatography with good reproducibility. The chromatographic solvent which was developed gave a good separation of sugars which was comparable to that by the *n*-butanol–NH_3 (1 % w/v) solvent of PARTRIDGE AND WESTALL[20]. The percentage of recovery was 90.8 for sucrose and 99.3 for glucose. SHALLENBERGER AND MOORES[11] found 93.7 % recovery of sugars in the 10–200 μg range. The high percentage of recovery of sugars from paper chromatograms is because only 1 % sugar was lost during chromatography due to lag or absorption between the original spot and the final spot (KOWKABANY AND HORDIS[21]). Percent error of the present method varied from 1.6 for ribose to 2.9 for glucose. This is in the order of the 2 % error of the Somogyi-Nelson copper colorimetric method assessed by SHU[22].

Ion exchange resins are commonly used for partial purification of sugar extracts. WILLIAMS *et al.*[23, 24] studied the use of resins for this purpose and found the resins were superior and more convenient than the old lead precipitation procedure. However, HULME[25] warned against the use of strongly basic anion exchange resins which may decompose the sugar to lactic acid. Amberlite IR 45 (OH^-) adsorbed the organic acids which could be eluted with a volatile acid and were suitable for organic acid analysis. Dowex 50 (H^+) adsorbed the amino acids which could be eluted with 10% NH_4OH and were suitable for amino acid analysis. Thus, this method of sugar analysis fitted into a general analysis of plant material.

The method described in this paper could handle a large number of determinations such as are often required in plant analysis. This facility was due in large part to the efficiency of the micro-elution tube. It allowed a small volume of eluent to be used for complete elution which made possible the micro-colorimetric determination directly in the test tube. It eliminated certain problems of other elution procedures (DIMLER *et al.*[12]), the problem of extraction (DUFF AND EASTWOOD[8], WHISTLER AND HICKSON[9] and DUBOIS *et al.*[10]), and the blank correction (SHALLENBERGER AND MOORES[11]). The micro-elution tube unit could be used for the elution of other chromatographed substances on paper, providing suitable eluent is used. It was easily constructed from ordinary laboratory materials.

SUMMARY

A simple quantitative procedure for sugar analysis of plant materials by paper chromatography with good reproducibility is described. The alcoholic extract was partially purified by a tertiary solvent system and ion exchange resins and then paper chromatography with the following solvent: water-saturated *n*-butanol, 95 % ethanol and trichloroethylene (6:2:2, v/v). The sugars were selected by a guide strip technique, and eluted from the "cut-out squares" of paper with 3 ml distilled water by a micro-elution tube, here described. They were determined by a microcolorimetric method. The method permitted 72 determinations to be done at one time.

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