PAPER CHROMATOGRAPHY–ANTHRONE DETERMINATION OF SUGARS*

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INTRODUCTION

Paper chromatography has been used as a tool in the determination of sugars since the original publication on the subject by PARTRIDGE AND WESTALL[1] appeared in 1948. In many cases the sugars have been located on the paper and determined without elution using photometric[2], reflectance[3,4] or visual[5] measurements. Another technique which has been used is to separate the sugars on paper, locate the sugar spots by means of a color reagent, elute the spots and measure the color spectrophotometrically. Among the reagents which have been used to develop the color using this technique are *p*-anisidine hydrochloride[6] and aniline hydrogen phthalate[7]. PHILLIPPU[8] and SHALLENBERGER AND MOORES[9] have described methods which involve location of sugars on the chromatogram, elution of the unsprayed spots, development of the color with suitable reagents and spectrophotometric analysis of this color.

The anthrone reagent has been used extensively in carbohydrate analysis since the original report by DREYWOOD[10] in 1946. This reagent is a general reagent for carbohydrates and is usually used for the determination of total sugars. However, WISE *et al.*[11] have shown that the anthrone reagent may be used at 50° for the determination of total fructose. Since sugars may be quantitatively eluted from paper using water[12], it would seem that a procedure utilizing paper chromatographic separation followed by elution and anthrone analysis would be a rapid and reliable method for the determination of sugars. Some work centered around these procedures has been reported by DIMLER *et al.*[13], WHISTLER AND HICKSON[14], PAVLINOVA[15] and TAKI[16]. In this paper the standardization of a procedure using paper chromatographic separation of glucose, fructose and sucrose and their subsequent elution and analysis employing the ROE anthrone reagent[17] is reported. The use of this method in the determination of these sugars in plant tissues is also reported.

EXPERIMENTAL

Apparatus

The chromatograms were developed by ascending and descending techniques in pyrex chromatographic jars (8½ × 18 and 12 × 24 in. respectively). The chromatographic paper used was Whatman No. 1 filter paper, which was shown to contain no water extractable substances that interfere with the anthrone reagent.

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The color formed with the anthrone reagent was read in an Evelyn colorimeter (Rubicon Co., Philadelphia, Pa.) using a 620 mμ filter.

To obtain reproducible spotting volumes, a microsyringe pipet control and micropipets (Hamilton Co., Whittier, Calif.) were employed.

Reagents and solutions

Three stock solutions containing 200 mg of glucose, fructose and sucrose respectively per 10 ml of 80 % ethanol were prepared. A fourth stock solution containing 200 mg each of glucose, fructose and sucrose per 10 ml of 80 % ethanol was prepared.

The developing solvents were those suggested by DURSO AND MUELLER[18]. Ethyl acetate, acetic acid and water were used in the ratios (v/v) 6:3:2, 5:3:2 and 4:3:2. The solvent was freshly prepared once a week.

The spray reagent used was prepared by dissolving 1.0 g of *p*-anisidine hydrochloride in 10 ml of methanol and diluting to 100 ml with 1-butanol. To this solution was added 1.0 g of sodium hydrosulfite. After shaking for several minutes, the mixture was filtered and stored at 4°. The reagent became somewhat colored but was suitable for use for several months.

The anthrone reagent[17] was prepared in the following manner: concentrated sulfuric acid (333 ml) was slowly added to 140 ml of distilled water. Care was taken to prevent the temperature from rising above 110°. Five grams of thiourea (Baker analyzed) was then dissolved in this solution. After the solution had cooled to 90°, 0.25 g of anthrone (Mathieson Chemical Company) was added and the resulting solution stored at 4°. This reagent remained usable for 4 weeks.

Procedure

The solutions containing the sugars in 80 % ethanol were spotted on the paper using the microsyringe pipet control and micropipets. For maximum separation, the spots should not contain more than 300 μg of any sugar. In the analysis of unknown solutions, 10 μl of the fourth stock solution was spotted as a separate spot to serve as an internal standard for glucose, fructose and sucrose. This solution was also spotted on both ends of the chromatogram to serve as end strips in locating the sugars. After air drying, the chromatograms were developed by either descending or ascending techniques.

The spots were located in the following manner: both end strips were cut from the paper and sprayed with *p*-anisidine hydrochloride solution. After drying for 5 min, the strips were held about 3 in. above a hot plate until the color appeared.

Using these end strips as guides, the unsprayed portion of the paper was cut into squares each containing one of the separated sugars. These squares were cut into smaller pieces and placed into test tubes. The sugars were extracted with 5 ml of water (about 1.0 ml of water is used for each 20–50 μg of sugar). During the extraction process (about 1 h) the tubes were shaken every 7–10 min.

To remove the cellulose fibers from the mixture the following procedure was used: filter paper (9 cm Whatman No. 1) was folded into funnels and 50 ml of distilled water was run through the paper in order to remove loose fibers. After the filter papers had completely dried in the funnels (24 h at room temperature or 1 h at 100°), the mixtures containing the extracted sugars and paper strips were filtered through the

previously washed and dried filter papers. Usually a large number of filter papers were washed and dried and kept ready for use.

The analysis was carried out using 1 ml of the filtrate which should contain 20–50 μg of sugar. One ml of the filtrate was added to 10 ml of anthrone reagent in a test tube. The tubes were capped with rubber caps, shaken and then heated in boiling water (95°) for exactly 15 min. After cooling rapidly to room temperature (a cold water bath may be used), the samples were transferred to colorimeter tubes and read in the Evelyn colorimeter using a 620 mμ filter. A blank containing 1.0 ml of distilled water in 10 ml of anthrone reagent was run with each set of tubes. The spots resulting from the stock solution of glucose, fructose and sucrose were used as the standards.

In order to check the amount of recovery of the sugars from the paper, two sets of ten analyses were run. One set consisted of chromatographed sugars containing 200 μg of each sugar. Extraction of these sugars, after chromatography and elution with 5.0 ml of water, gave a solution which would contain 40 μg of sugar per ml if complete extractions occurred. These were run against unchromatographed solutions of the sugars obtained by diluting the stock solutions so that the concentrations were 40 μg/ml. Both sets were treated exactly the same and were run simultaneously.

The extracts of plant tissue (dried, pulverized corn seedlings were used in this investigation) were obtained by extraction of a known amount of tissue (1.0 g) with a known volume (10 ml) of 80% ethanol. After overnight extraction on a shaker at room temperature, the mixture was filtered and the filtrate used directly.

A check on the recovery of sugars from plant material was carried out. In this experiment, known amounts of the sugars were added to spots containing the plant extracts. The spots were developed and analyzed in the usual way and the results compared with those from the spots which are not spiked with additional sugars. A chromatographed standard was also run in these recovery experiments.

RESULTS

The best separation of a mixture of glucose, fructose and sucrose occurred using descending techniques. The best solvent system used was ethyl acetate–acetic acid–water (6:3:2). Using this solvent system and descending techniques, samples containing in excess of 200 μg of each sugar were easily separated. On the other hand, using ascending techniques, reliable results could not be obtained using more than 100 μg of each sugar.

The reproducibility of the descending method is excellent as is shown in Table I. Since the samples were usually run on different days employing a different anthrone reagent, or one of different age, the optical density was not directly proportional to the amount of sugar between different runs. That is, the optical density for 40 μg of glucose analyzed on one day may not be twice that for 20 μg of glucose run on a different day. A chromatographed standard should be run with each analysis to compensate for differences in anthrone reagent and any differences in handling.

The percentage recovery of the sugars from paper is shown in Table II. The recovery of the separated sugars was quite good except for sucrose. However, the value of 84% recovery was not considered to be critical since a chromatographed standard of about the same concentration as the unknown was always run. This spot then served as a standard for the colorimetric analysis.

TABLE I

REPRODUCIBILITY OF THE PAPER CHROMATOGRAPHY–ANTHRONE DETERMINATION OF SUGARS USING THE DESCENDING TECHNIQUE

Sugar	µg/spot	Optical density	
		Mean*	Std. dev.
Fructose	100	0.1215	0.0113
	200	0.311	0.014
Glucose	100	0.1006	0.0049
	200	0.1700	0.0040
Sucrose	100	0.1193	0.0042
	200	0.2518	0.0041

* Results of 12 analyses. A different anthrone reagent or one of different age was used for each set of 12 analyses. Therefore, the optical density was not directly proportional to the amount of sugar between different runs.

TABLE II

RECOVERY OF SUGARS FROM PAPER

Sugar (200 µg/spot)	Trial	Source	Optical density		Recovery (%)
			Mean*	Std. dev.	
Fructose	1	Stock solution	0.301	0.005	
		Eluted from paper	0.312	0.010	103.6
Fructose	2	Stock solution	0.2722	0.0035	
		Eluted from paper	0.2907	0.0116	106.8
Glucose	1	Stock solution	0.1968	0.0043	
		Eluted from paper	0.2096	0.0036	106.5
Glucose	2	Stock solution	0.1997	0.0027	
		Eluted from paper	0.2151	0.0090	107.7
Sucrose	1	Stock solution	0.2359	0.0047	
		Eluted from paper	0.1966	0.0022	83.3
Sucrose	2	Stock solution	0.2455	0.0047	
		Eluted from paper	0.2001	0.0064	84.0

* Results of 10 analyses. Different anthrone reagents were used in each trial. Therefore, the optical density varied between trials.

TABLE III

COMPARISON OF THE PAPER CHROMATOGRAPHY–ANTHRONE METHOD WITH THE MODIFIED GLUCOSE-OXIDASE METHOD IN THE ANALYSIS OF CORN SEEDLINGS

Sugar	Sugar content (mg/g)		Recovery data	
	Paper chromatography–anthrone method	Glucose-oxidase method	Sugar added (µg)	Sugar recovered (µg)
Fructose	73.2	75.8	100	98.9
Glucose	50.5	48.0	100	94.8
Sucrose	225.6	230.1	100	111.6

The method was applied to analysis of glucose, fructose and sucrose in dried corn seedlings. The results obtained were compared with those obtained from the same samples using a modified glucose-oxidase method[19]. Table III shows that the paper

chromatography–anthrone method compares favorably with the modified glucose-oxidase method. In the analyses of plant extracts it is necessary to use chromatographed standards and to run triplicates of the plant extracts. It is not necessary to run an internal standard on each chromatogram. The spots from many chromatograms may be analyzed using the internal standards from another chromatogram, provided all samples and standards are analyzed simultaneously using the same anthrone reagent.

Table III also shows that sugars added to plant extracts may be recovered in good yields. Since the recovery of the chromatographed standard was taken at 100 %, the recovery of sucrose appears to be greater than 100 %. This is not critical if the internal standard contains approximately the same amount of sucrose as the material analyzed. A preliminary analysis to determine the approximate amount of sugar in the unknown should be carried out before more exact analyses are run. In the case of glucose and fructose this is not so important since the recovery of these sugars from paper has been demonstrated to be approximately 100 % (Table II).

The method, when used according to the described technique, gives reliable results. It is possible to analyze a large number of samples rapidly using very simple apparatus. Once the technique has been mastered, 20 samples per 24 h period may be analyzed for glucose, fructose and sucrose. The method can be readily adjusted to include any sugars which can be separated by paper chromatography.

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SUMMARY

Mixtures of glucose, fructose and sucrose were successfully analyzed by a method employing separation on paper, elution and colorimetric determination with the anthrone reagent. Since the anthrone reagent is employed as the colorimetric reagent, prior hydrolysis of sucrose is not necessary. The method, which requires very simple apparatus, may be used for the rapid and accurate determination of sugars in plant materials.

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