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QUANTITATIVE RECOVERY OF SUGARS FROM SILICA GEL THIN LAYERS

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SUMMARY

Simple equipment and techniques were used for quantitative thin-layer chromatography and for recovery of sugars by elution. Although sugar recovery was not complete and varied quantitatively with the type of silica, it was consistent with a single silica and could be measured with good precision. The mean recovery of D-glucose was 95.2% with a relative standard deviation of 0.0074. The extent of losses of many sugars was quite similar. The losses occurred rapidly in conjunction with the drying of sugars on silica gel.

INTRODUCTION

The usefulness of thin-layer chromatography (TLC) for quantitative work will not be fully realized without further development of rapid and simple techniques that maintain good precision. This was the objective sought here. Although this report is limited to the recovery of sugars after TLC on silica gel, the techniques have broader applications. The precision reported here for sugar recoveries could be equaled or exceeded with other substances because the limitation of precision was due more to the sugar analyses than to TLC. This study does not include a comparison of mobile phases for chromatographic separations of sugars.

A review of elution techniques in quantitative TLC, with particular attention to precision, has been written by COURT[1].

METHODS

The basic procedure used in this work for the collection and elution of samples was described by MILLETT *et al.*[2]. Several simplifications of this procedure as well as special techniques for sugars were developed and are described.

* Maintained at Madison, Wisc., in cooperation with the University of Wisconsin.

Plate preparation

Thin-layer plates (20 cm × 20 cm) were prepared with a spreader that provided an even-layer wet thickness of 250 μ. Unless specified otherwise, the adsorbent was SilicAR-7*, a silica without binder. The dried plates were divided into lanes with a plate scriber (a rigid plastic sheet, 24 cm × 7 cm × 0.6 cm, notched on one long edge). The scriber formed alternate 2.2-cm and 1.4-cm lanes on the plate with separations of 1.5 mm where adsorbent was removed. To ensure uniform lane lengths, excess silica was removed from the top edge of the plate. Guides for the starting line were provided by dotting the edges of the wide lanes at a distance of 3 cm from the bottom edge of the plate.

Bureau of Standards D-glucose was used for all glucose measurements. Other sugars were commercial samples, some of which were purified by preparative TLC. Samples were applied from a 2-μl micropipette** to the 2.2-cm lanes in a series of three or four applications across each lane. The sample then approximated a streak $^1/_2$ cm or less broad. A single reference was applied to the center of each narrow (guide) lane at the starting point.

A few precautions were taken to assure uniform delivery from micropipettes. A single micropipette was used for most of the determinations and was stored in distilled water. It was occasionally rinsed with dichromate cleaning solution. Before sample application, the sidewall of the filled micropipette was held against a piece of filter paper and rolled to remove sample clinging on the outside of the micropipette. The pipette was then examined to ensure that it was completely full.

Chromatography

The mobile phase for most chromatograms was a mixture of ethyl acetate–acetone–glacial acetic acid (6:3:1). Plates were placed in an unequilibrated chamber a few minutes after the mobile phase was added. Chambers were kept in small insulated boxes at room temperature (about 23°). Plates were not removed until 5 to 10 min after the slowest lane had completely developed, usually a period of 70 to 100 min. The occasional fast lane was noted because spot migration was slightly increased in these cases.

Detection

Aniline phthalate solution (800 ml of butanol, 40 ml of water, 8 ml of aniline, 14.7 g of phthalic acid) and heat were used for detection of spots on the developed guide strips. The solution was streaked down the centers of guide strips with a 0.1-ml syringe fitted with a very fine-bore, flexible plastic needle. A simple wood slide and guide similar to that described by McKIBBINS *et al.*[3] was used to apply about 0.013 ml of reagent to a guide strip.

To heat narrow zones on the guide lanes, a resistance wire inside a small-bore glass tube was fixed to a holder that allowed the glass tube to be laid directly on the silica. Spots appeared after 20 sec of electrical heating.

The positions of developed samples were then located by reference to adjacent guide strips. Ordinarily an area extending 1.5 cm on each side of the assumed spot center was marked for removal (about 7 cm^2).

* Mallinckrodt Chemical Works, 3600 N. 2nd. Street, St. Louis, Mo. 63160.
** "Microcap", Drummond Scientific Co., 524 N. 61st. Street, Philadelphia, Pa. 10151.

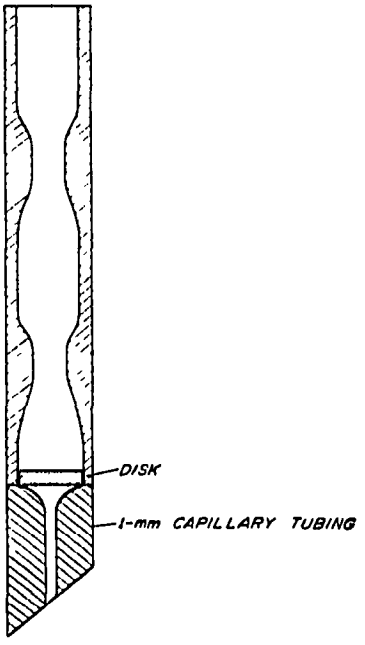

Fig. 1. Pickup tube for removing silica from thin-layer plates and for subsequent elution of adsorbed samples.

Removal of samples from chromatograms

Pickup tubes (Fig. 1) for removing silica were prepared from 5-mm-bore glass tubing as follows: A medium-grade, fritted glass disk was cautiously sealed inside the tubing and about 1 cm of 1-mm-bore capillary tubing was sealed on just below the disk, leaving a minimum volume between disk and capillary. The capillary tubing was cut off with a diagonal cut and was fire-polished. The 5-mm tubing was slightly constricted at 1 cm and at 3 cm above the disk and was cut off at 4.5 cm above the disk. The small fritted glass disk was not easily sealed within the glass tubing without excessive fusion. Its final porosity when tested with water should permit emptying the pickup tube (about 0.8 ml of water) in 8 to 40 sec under a net pressure of about 580 mm of mercury.

A 2.5- to 3-cm length of thin-walled polyolefin tubing that snugly fit into the pickup tube was pulled down at one end to about 1.5-mm diameter and was cut off diagonally. This plastic tip when inserted into the pickup tube easily picked up the adsorbent with suction.

A satisfactory pickup tube similar to that used by MILLETT *et al.*[2], but constructed from polyethylene tubing, is easily made with an integral tip; however, its asbestos mat requires more preparation time than does cleaning the counterpart fritted glass disk by backwashing. The fritted glass disk should be flushed soon after use. If it becomes clogged, it can be restored by soaking in alkali. Silicas with binder are slightly more difficult to remove from the pickup tubes and from thin-layer plates than are silicas without added binder.

Elution

After a sample was collected, the pickup tube was tapped to settle the bed of

silica into the bottom of the tube, and a few small drops of water were added slowly to wash the sides of the tube. A small-bore, flexible plastic needle attached to a small syringe was especially satisfactory for adding the water. After the first 0.05 to 0.1 ml of water had seeped into the silica, the tube was filled with water from the syringe. Elution time was often shortened to 10 to 15 min by a small amount of pressure.

Each eluate was collected in a 0.1-ml micropipette (Microcap). This step was facilitated by clipping pickup tubes in a vertical position onto a wooden holder. The horizontal base of the holder had narrow slots in which the 0.1-ml pipettes rested.

After filling, each 0.1-ml collection pipette was emptied into exactly 0.9 ml of water in a screwcap vial. A micropipette holder (Drummond) was used to blow out the 0.1-ml pipette and to suck up some of the sample solution for two or three rinsings. The micropipette holder consisted of 4 cm of glass tubing with a punctured rubber plug (to hold the micropipette) at one end and a punctured rubber medicine-dropper bulb at the other end.

Spectrophotometric measurement

For a series of samples, the 1.0 ml volume per sample was sufficient for rinsing and for finally filling a 0.25-ml syringe. The analysis of the 0.25-ml aliquot by dehydration with 2.0 ml of sulfuric acid followed by spectrophotometric measurement has been described[4]. For this work, there were two minor changes: (1) Reagent grade concentrated sulfuric acid was used without adjusting its concentration to 95.0% and, (2) the 70° water bath for reaction tubes was replaced by a heating block. A 30-min heating period was used as before.

Because this analysis can easily detect contamination such as cellulosic dust, a few simple but necessary precautions were taken. It was most convenient to dispense the concentrated sulfuric acid from a 9-lb. reagent bottle into a 100-ml glass-stoppered bottle by means of a glass hand pump* screwed onto the reagent bottle. The delivery tip of the pump was protected by a glass vial when not in use and was rinsed with water before using. If acid is poured from a reagent bottle, care must be taken not to collect drips from the side of the neck. The open ends of the reaction tubes (16-mm × 150-mm test tubes) were cleaned in dichromate cleaning solution, rinsed, and covered with clean glass vials; during this procedure handling of the lip areas was always avoided. The tubes were continually covered and protected from dust during use and during storage. The covered tubes were dried in a test-tube rack by laying the rack on its side in a clean oven. Excessive dust within the oven can cause erratic blanks.

For each spectrophotometric run, three blanks of 0.25 ml of water in 2.0 ml of H_2SO_4 were prepared. The absorbance of reagent blanks, depending on the acid, varied from day to day from about 0.012 to 0.022 at 320 nm, but the range within a set of three was usually within 0.004 when the reaction tubes were clean.

Plate blanks were obtained by removing silica samples from blank lanes. These blanks were found to vary from 0.001 to 0.008 with an overall average of 0.004, which was consistent enough to be used without measurement at each run. This blank may change for different silica gels and possibly for different batches of the same silica. SPENCER AND BEGGS[5] found that the blank due to very small silica particles can be reduced by membrane filtration. The blank is here reduced by dilution and by fil-

* "Repipet", Cole-Parmer Instrument Co., 7425 North Oak Park Avenue, Chicago, Ill. 60648.

tration through the fritted glass (or asbestos mat). To increase the sensitivity of this method, it would be necessary to further reduce blanks by some means such as membrane filtration and perhaps also by special extraction of the silica and purification of the solvents used for development.

Standard 2-μl samples were applied onto 4-mm squares of Whatman No. 1 filter paper in screwcap vials. These samples were diluted with 0.9 ml of water, and the net absorbance from the reaction of a 0.25-ml aliquot with 2.0 ml of H_2SO_4 was corrected to a sample volume of 1.0 ml. The preparation of the paper squares included a vacuuming with the tip of a medicine dropper attached to a vacuum line. After removing cellulosic dust in this way, the residual paper blanks varied from 0.001 to 0.006. An average of 0.003 was taken as a uniform paper blank.

The recovery of a sample removed from a thin-layer plate is expressed as a percentage of its mean standard by comparing net absorbances after corrections for blanks.

RESULTS AND DISCUSSION

Application of samples

FAIRBAIRN[6] discussed the critical importance of sample application in quantitative chromatography and reported the results obtained with several devices. He stated his best results were obtained with a mechanical applicator. Here it was found that the precision of the 2-μl micropipette was considerably better than the 3.3% relative standard deviation reported by FAIRBAIRN and the 1.5% reported by JORK[7]. In a set of nine replicate glucose standards, applied and measured as described, the relative standard deviation was 0.55% at a mean absorbance of 0.872 (about 65 μg of glucose). In measurements on 6 different days, a total of 21 samples had a mean absorbance of 0.811 and a relative standard deviation of 0.59%.

Precision of sugar recovery

The recoveries of glucose samples applied to silica gel layers, but not chromatographed, were measured by removing and eluting about 7 cm^2 of silica including the sample. The mean recovery of 25 of these samples in seven runs was 98.1% of the mean standard sample. The relative standard deviation of these recoveries was 0.0091. This includes variation between and within runs.

The chromatographic step was similarly tested by comparison between chromatographed and standard samples. The mean recovery of 23 samples from six runs was 95.2% with a relative standard deviation of 0.0079.

An efficient determination consists of three replicate chromatographed samples (when an average plate blank and an average percentage recovery are known). The chromatographic data were recalculated to find the day-to-day variability between means of three samples. This variability had a relative standard deviation of 0.0074 at the 95.2% mean recovery value (Table I).

Variability between samples within a single run was computed from chromatographed (mean recovery, 95.2%) and unchromatographed (mean recovery, 98.1%) samples. This variability (Table I, line 3) was less than that between chromatographic runs on different days (line 4). The additional error between days was in the chromatographic step because the standards did not show the same effect. Although the smaller

TABLE I

VARIABILITY OF GLUCOSE DETERMINATIONS

Samples	*Data points*	*Standard deviation*	*Relative standard deviation*
Standards	9 (1 run)	0.0048[a]	0.0055
Standards	21 (6 runs)	0.0048[b]	0.0059
Applied to silica	48 (13 runs)	0.54[c]	0.0056
Chromatographed	23 (6 runs)	0.75[d]	0.0079
Chromatographed	23 (6 runs)	0.71[e]	0.0074

[a] Based on a mean absorbance of 0.872.
[b] Based on 21 samples with a mean absorbance of 0.811.
[c] Variability between samples within a single run; based on percentage recovery.
[d] Includes variability within and between runs, and variability around an average recovery of 95.2%.
[e] Computed variability of the means of three samples between runs; overall mean recovery, 95.2%.

error might be used by measuring recoveries of standards and unknowns in a single run, the 0.71 standard deviation between means of three samples is considered the more practical result. Thus, after correction for expected loss on the plate, the mean recovery value of three 60-μg samples of glucose had a 95% probability of being within 3% of the applied amount.

The glucose samples used for determination of recovery had a net absorbance of about 0.8. A considerable part of the variability, that part due to reagent and plate blanks, was independent of sample size and consequently caused greater errors for smaller samples. This was shown by mean percentage recoveries of 20 μg of glucose in three runs that had standard deviations of 2.7 (three samples) and 2.5 and 3.6 (five samples each) compared with the 0.534 standard deviation within a day found for 60-μg samples.

The attainable precision of recovery from silica layers is probably better than that found here with 60-μg glucose samples. The similarity of the variability of standards (Table I, lines 1 and 2) and the variability within chromatographic runs (line 3) is an indication that limitations were imposed by factors not in the chromatographic step. Other colorimetric measurements, reduction of blank values, or classes of compounds other than carbohydrates may result in less variability, especially with small sample sizes. The precision reported here is very similar to the 0.74 to 0.91% relative standard deviation obtained by MILLETT *et al.*[2] for 25 to 100 μg of furoic acid recovered after TLC.

Quantitative analysis

Although glucose samples were not completely recoverable after chromatography, the uniformity of recoveries permitted using a correction factor, 1.05, for estimating the amount of glucose applied to the plate. The reliability of this factor was only well established with 60 μg of glucose, but the results in Fig. 2 suggest that it could be used for a wide range of sample sizes. The 95.2% average recovery of glucose was confirmed by recovery of the same percentage of radioactivity after TLC of a

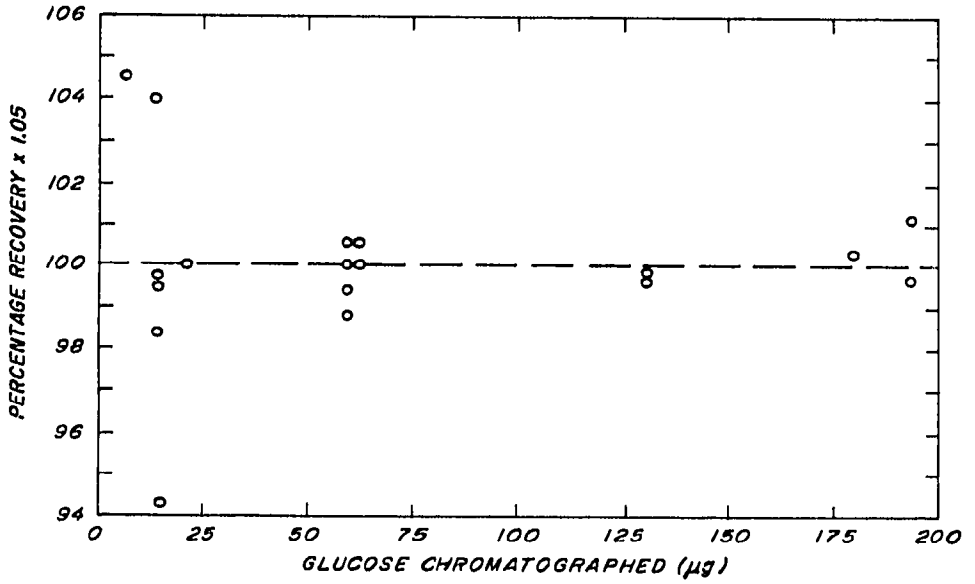

Fig. 2. Variability of corrected glucose recoveries (means of three samples) after thin-layer chromatography on silica gel.

single sample of [^{14}C]glucose. The [^{14}C]glucose used for this test was previously chromatographed by TLC and recovered.

The scatter of results in Fig. 2 at 10 to 20 μg of glucose is evidence of the larger relative errors with these smaller amounts of glucose. To decrease this error, it is practical to increase the sample by two or three applications from a 2-μl micropipette. The larger samples in Fig. 2 were applied in this way.

Insufficient data were collected to determine correction factors for other sugars with the same reliability as for glucose. However, the means of a few determinations were within the limits of error around the 95.2% recovery of D-glucose (L-arabinose 94.8%, D-xylose 96.1%, D-galactose 94.7%, D-mannose 94.4%, sucrose 96.9%).

About 1 h was required for application, detection, pickup, and elution of three replicates. A total time of 3.5 h included also 90 min for development and 45 min for the analytical steps after elution.

Nonrecoverable sugar

There was evidence that the losses of sugar occurred very abruptly at the moment of drying of samples on silica. The recovery of xylose from unchromatographed layers of Silica Gel H was found to be 94% after a few minutes of drying, 91% after 1 h, and 84% after 17 h. However, when a plate was put into a closed chamber at 100% relative humidity immediately after sample application, the recovery was 99% after 1 h in the chamber. There was visual evidence of material left at the starting point of sprayed plates and also at the first position of migration when double development was used. COXON[8] has also reported spots left at the start of a chromatogram after TLC of glucose.

Direct evidence of strong adsorption of glucose was obtained from a spectrophotometric measurement which showed about 1% recovery of hexose in the water eluate of five start areas after TLC of a total 600 μg of glucose. The possibility of extra spots must be considered during qualitative interpretation of multiple-developed

chromatograms and argues against the use of multiple development for quantitative purposes.

Properties of the adsorbent

Initial work with Silica Gel H led to the finding of losses as mentioned and to the testing of other silicas. A comparison based on the percentage recovery of xylose after chromatography gave the following recoveries: Silica Gel H 93.1%, Silica Gel GF 94.6%, and SilicAR-7 96.0%. When xylose was applied, the recoveries without chromatography were the following: Kieselguhr 97.7%, Silica Gel GF 94.3%, Silica Gel H 90.3%, and SilicAR-7 96.6%. Other tests with glucose gave variable results that indicated Silica Gel H gave almost as good recoveries as SilicAR-7 after chromatography but less recovery without chromatography. SilicAR-7 was chosen for further work because recoveries from it were high and consistent. In any case, it appears that different brands of silica gel may give different recoveries, and that different batches of a single brand may possibly give different recoveries.

Other adsorbent factors tested with SilicAR-7 were found of lesser importance than the kind of silica. The drying time of the applied sample or of the chromatographed plate was not critical up to 75 min, the length of the test, which is more than sufficient time for the detection and removal of samples from two or three plates.

Activation of plates at 110° or 150° did not change percentage recoveries of glucose from that found for a plate dried at room temperature, although development time was shorter and separations were improved on the drier plates.

The area of developed spots was estimated by removal of 1- and 2-cm bands of silica. All of the expected glucose and 97% of the expected xylose samples were contained within 2-cm bands. About 80% of the glucose and 70% of the xylose were contained within 1-cm bands. The glucose spot center migrated about 3 cm and the xylose spot center about 7 cm. About 60-μg samples were used for these tests. Because of potential error in spot location, a practical lower limit of separation of spot centers for quantitative determinations would be 3 cm in the system described. With care, 2.5-cm separation could give useful data.

The solvent system used in this work spread galacturonic acid over a large area. However, glucurone that migrated 13.5 cm was contained in a 2-cm band and 4-O-methylglucuronic acid that migrated about 11.5 cm appeared to be equally well concentrated.

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