

R60-36

ANALYTICAL BIOCHEMISTRY 2, 133-140 (1960)

TECHNICAL LIBRARY  
QUARTERMASTER RESEARCH &  
ENGINEERING CENTER  
NATICK, MASS.

## Micro Column Chromatographic Method for Analysis of Oligosaccharides

GAIL LORENZ MILLER

*From the Pioneering Research Division, Quartermaster Research and  
Engineering Command, United States Army, Quartermaster Research  
and Engineering Center, Natick, Massachusetts*

Received April 21, 1960

### INTRODUCTION

The successful resolution of malto- and cellodextrins of different degrees of polymerization by ethanol-water gradient elution from chromatographic columns composed of stearic acid-treated mixtures of charcoal and Celite (1, 2) suggested the development of a micro analytical method based on these principles. The method, described below, was worked out with the aid of cellodextrins of degree of polymerization of 2-7 and of selected oligosaccharides of other series. It provides means for qualitative identification of individual oligosaccharides and quantitative measurement of their concentrations. One of its more important uses might be in kinetic studies on the breakdown, or formation, of different polysaccharides.

### EXPERIMENTAL

#### *Adsorbent*

Forty grams of Darco G-60<sup>1</sup> and 40 gm of Celite 545<sup>2</sup> were mixed with 320 ml of 2½% stearic acid (technical) in absolute ethanol for ½ hr, followed by filtering with suction. The adsorbent mixture was then suspended in 320 ml of 50% ethanol saturated with stearic acid, refiltered, resuspended in 200 ml of water, and refiltered again. The final product was dried to a powder in a vacuum desiccator over calcium chloride.

#### *Test Materials*

The cellodextrins used were prepared from acid hydrolyzates of cellulose (2). Individual cellodextrins were dissolved in water to give concentrations of 0.1%. In special experiments 1% concentrations also

<sup>1</sup>An activated carbon preparation manufactured by Atlas Powder Company, Wilmington, Del.

<sup>2</sup>A siliceous filter-aid manufactured by Johns-Manville Co., New York, N. Y.

were used. Solutions of mixtures of cellodextrins were made up to contain 0.1% glucose, 0.1% cellobiose, and 0.1% concentrations of each of other homologs of the series up to and including celloheptaose.

Laminaribiose, laminaritriose, laminaritetraose, isomaltose, and isomaltotriose, obtained from enzymic hydrolyzates of laminarin and dextran, were supplied by Dr. E. T. Reese. A preparation of maltose which was tested was obtained from Central Scientific Company, New York.

#### *Preparation and Use of Column*

Five hundred milligrams of dry adsorbent mixture, prepared as described above, was placed in a fritted-glass micro filter tube which was 5.5 cm long, 1.0 cm inside diameter, and medium-coarse porosity. To wet

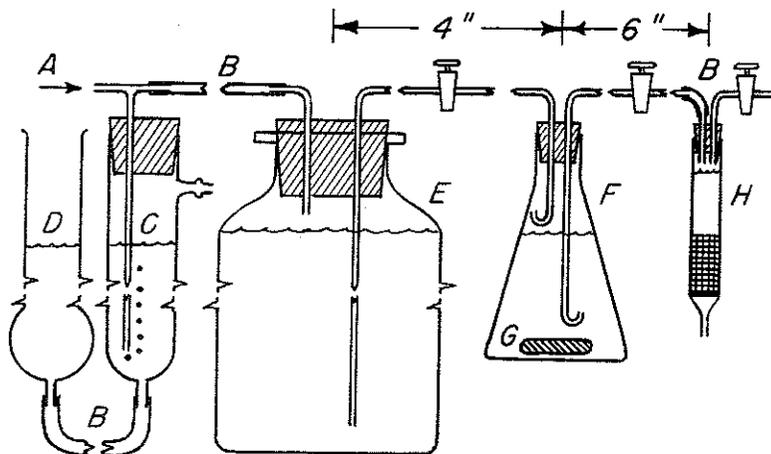


FIG. 1. Micro column chromatography apparatus. *A*, compressed air inlet. *B*, flexible rubber tubing connections. *C*, modified side-arm test tube containing water, used as a manostat. *D*, drying tube used as leveling bulb for manostat. *E*, 500-ml bottle used as reservoir for absolute ethanol. *F*, 50-ml Erlenmeyer flask used as mixing vessel. *G*, Teflon-covered magnetic bar operated by magnetic stirrer, not shown. *H*, elongated fritted-glass micro filter tube, used to contain column of adsorbent. The stoppers are rubber.

the adsorbent, it was suspended in 2–3 ml of 20% ethanol. The liquid was pushed through the adsorbent by blowing through a rubber tube connected to the top of the filter tube, and ethanol which remained on the adsorbent was removed by washing the column five times with 0.5-ml portions of water.

A 0.2-ml sample of individual mono- or oligosaccharide, or of mixture

of oligosaccharides, was introduced into the absorbent, followed by 0.1 ml water. Two milliliters of water was then layered above the adsorbent, prior to commencement of the elution. For the elution, the top of the column was connected to a 50-ml Erlenmeyer flask used as a mixing vessel, as shown in the diagram of the apparatus in Fig. 1. The mixing vessel, charged with 50 ml water and operated with the aid of a magnetic stirrer, was fed from a reservoir containing absolute ethanol. Air pressure, equal to a few centimeters of water, was applied to the system with the aid of a manostat. For convenience in starting and stopping the elution process and in charging the various components of the system, stopcocks were placed in the line between the column and the mixing vessel, between the mixing vessel and the reservoir, and on a vent at the top of the filter tube. The apertures of inlet and outlet tubes to the mixing vessel were each bent upward to prevent bubbles from entering the tubes. The aperture of the inlet tube was open to an air space above the surface of the liquid in the vessel.

The eluate from the column was collected in  $13 \times 100$  mm tubes with a drop-counting type of fraction collector, each tube receiving 10 drops. The pressure was adjusted to produce an initial rate of elution of one drop per 60 sec, and was not further adjusted. About 16 hr was required for elution of the most slowly eluted oligosaccharide tested, namely, celloheptaose.

#### *Analysis for Carbohydrate*

The entire eluate obtained for each fraction was used in the analyses for carbohydrate content. The fractions were each mixed with 3-ml of 0.2% orcinol in 70% sulfuric acid (v/v), heated for 20 min at  $100^\circ$ , and cooled to room temperature. Absorbances of the colored solutions which resulted were measured at a wavelength of  $550 \text{ m}\mu$  with the aid of an automatic recording colorimeter (3). The values of absorbance which were obtained were compared with those of similarly treated glucose standards consisting of duplicate samples of 0.0, 0.05, 0.10, and 0.15 mg glucose, each in 0.5 ml water. The amounts of carbohydrate in each eluate could then be calculated in terms of glucose, when desired.

An important prerequisite for good results was the strict exclusion of dirt and dust from fraction collector tubes and from reagent.

## RESULTS AND DISCUSSION

### *Characterization of Elution*

Because of surface tension effects, sizes of drops of eluate diminished as the concentration of ethanol increased. The magnitude of this effect

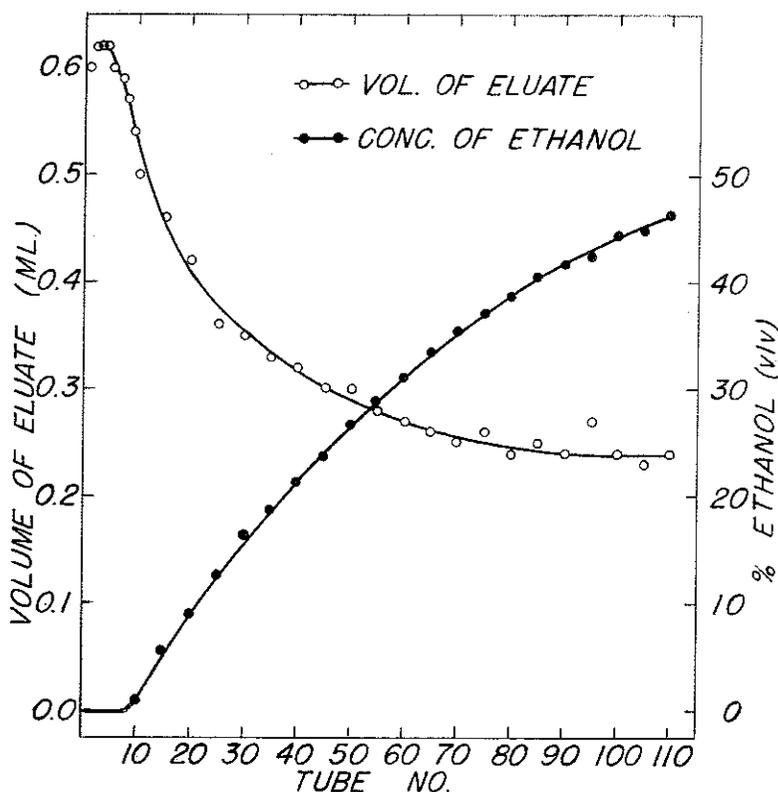


FIG. 2. Relation of volume of eluate and concentration of ethanol to tube number of eluate.

is shown in Fig. 2 by the relation of volume of eluate and of concentration of ethanol to tube number of eluate. Concentration of ethanol was measured refractometrically.

#### *Qualitative Aspects of Method*

Distribution patterns for individual cellodextrins and for a mixture of cellodextrins, in which values of absorbance are related to tube numbers of eluates, are shown in Fig. 3. The degrees of polymerization of the different components are indicated alongside the corresponding peaks in the patterns. It may be seen that individual oligosaccharides separated at the same tube numbers when tested in the presence of homologs as in their absence. In replicate tests it was found that positions of peak maxima seldom varied more than  $\pm 1$  tube. The concentrations of ethanol at which the different components were eluted from the ad-

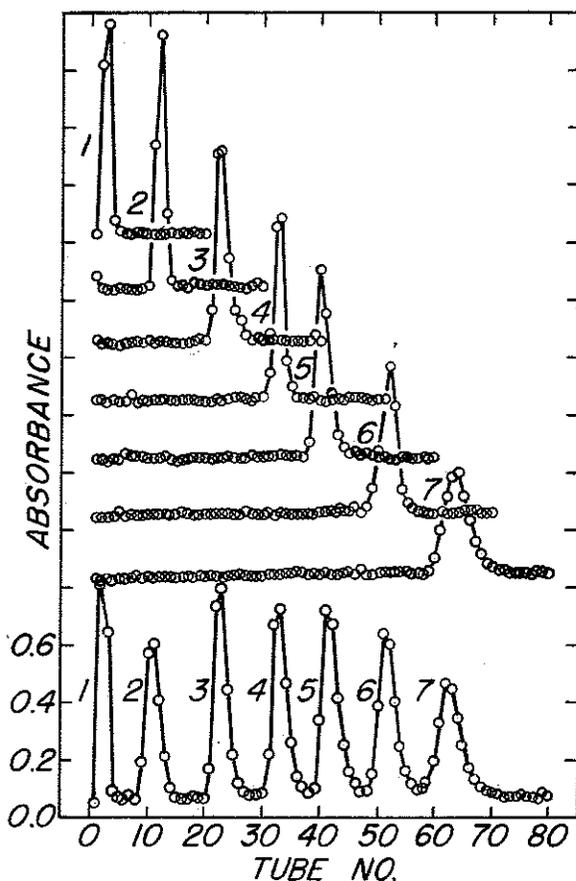


FIG. 3. Distribution patterns for individual cellodextrins and for a mixture of cellodextrins.

sorbent may be found by reference to Fig. 2. The slight "tailing" which was observed with each component could not be avoided.

Distribution patterns for laminaribiose, -triose, and -tetraose, not shown, revealed peaks at tube nos. 14, 27, and 40, respectively. Patterns for isomaltose and isomaltotriose revealed peaks at tube nos. 5 and 12, respectively. The commercial sample of maltose showed one major and two adjacent minor peaks; assuming the minor peaks to represent glucose and maltotriose, the peaks of the di-, and trisaccharide of this series appeared at tube nos. 8 and 15, respectively. It is clear from these results that resolution of homologs can be effected in series other than that of the cellodextrins and that corresponding isomers from different series may be distinguished from one another by differences in the tube

numbers at which they appear. The findings also suggest the possible use of the method for sugars differing in ways other than in degree of polymerization or in mode of linkage of monomeric units; for example, in length of carbon chain, in presence or absence of reducing groups, in nature of glycosidic substituent, in occurrence of aldose versus ketose groups, etc.

#### *Quantitative Aspects of Method*

The recoveries of glucose and of the different cellodextrins from the chromatographic column were found to be fairly quantitative when

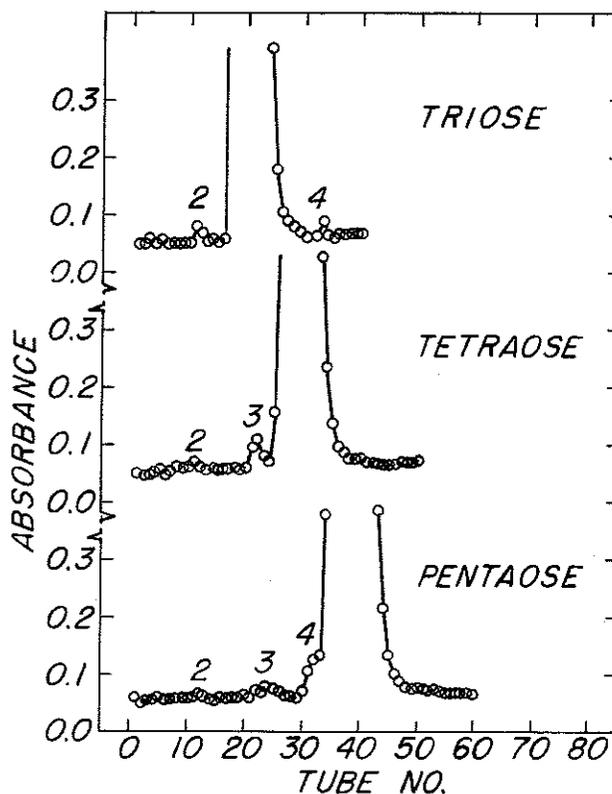


FIG. 4. Distribution patterns of 10-fold concentrations of cellodextrins, showing presence of homologs as contaminants.

appropriate corrections were applied to allow for differences in volumes of different eluate fractions, effect of ethanol on development of color, and uptake of water by the oligosaccharides as a result of hydrolysis in the color test. The best method of handling quantitative aspects of the

method, however, was by determination of empirical correction factors from data obtained by measurement of apparent recoveries of the different sugars, in terms of glucose. The factor found for glucose was 1.11; that for cellobiose, 0.94; those for cellotriose to -heptaose, 0.77. To calculate the true value for the concentration of cellotriose, for example, its apparent concentration in terms of glucose should be multiplied by 0.77. Results obtained by this procedure were accurate to  $\pm 5\%$ .

When cellotriose and -pentaose were added as artificial contaminants to cellotetraose in proportions of 2% of the total, they were readily detected on chromatographic analysis under the above conditions. Since homologs were not revealed as contaminants in the distribution patterns shown in Fig. 3, it could be concluded that contamination by any individual homolog was probably lower than 2%. More precise evaluation of contamination was possible, however, by analyzing the cellodextrins at 10-fold concentrations. Results obtained for cellotriose, -tetraose, and -pentaose under these conditions are shown in Fig. 4. It was calculated from the data that total contaminations with homologs was of the order of 1%, the principal contaminant in each instance being the next lower homolog. The more common occurrence of lower, rather than higher, homologs is explained by the nature of the tailing which occurred in the original chromatographic fractionation of the oligosaccharides (2). Had higher homologs been present in the different oligosaccharides, however, they would have been detected since it was found that cellopentaose added to cellotetraose in as low a proportion as 0.2% of the total sample could readily be detected. Similar analyses for cellohexaose and -heptaose, not shown, were somewhat less satisfactory. Excessive spread of the cellohexaose peak appeared to obscure any -pentaose present, while the low solubility of the -heptaose handicapped the significance of its analysis.

#### *Variables in the Method*

When charcoal-Celite mixtures were used which were untreated with stearic acid, the separation of oligosaccharide components from the column was retarded and the resolution was poor, confirming the original observations of Alm (1) in this respect.

The use of large test samples, or of more rapid elution rates, decreased the resolution of components, but did not change materially the numbers of the tubes at which the different peaks appeared. The use of steeper ethanol-water gradients or of shorter columns also decreased resolution, but at the same time accelerated the separation of components from the column and thereby affected the positions at which peaks appeared.

Reuse of columns led to tailing, doubling of peaks, and eventually to

slower rates of separation of components from the column. For best results, therefore, columns should not be reused.

Alteration of certain variables may be advantageous under special circumstances. For example, more rapid elution rates or steeper gradients may be used where only the qualitative aspects of the test are of importance. On the other hand, slower elution rates or shallower gradients may be used where greater separations of components are desired and where the longer period of time required for the test is not a serious drawback.

Different batches of adsorbent may show somewhat different chromatographic properties. To allow for this, a calibration could be carried out for a given batch of adsorbent, using a slowly eluted material such as cellohexaose as a reference material. The tube numbers at which other oligosaccharides appear may then be expected to be proportional to that at which the cellohexaose appears.

#### SUMMARY

A micro column chromatographic method for qualitative identification and quantitative measurement of oligosaccharides from cellulose and other series of oligosaccharides is presented. Analyses are made with 0.2-mg quantities of test material. Contaminants in the form of homologs are estimated with the use of 2-mg quantities.

#### REFERENCES

1. ALM, R. S. *Acta Chem. Scand.* **6**, 1186 (1952).
2. MILLER, G. L., DEAN, J., AND BLUM, R., *Arch. Biochem. Biophys.* In press (1960).
3. MILLER, G. L., AND CUMMINGS, C. L., *Anal. Chem.* **31**, 481 (1959).