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Gradient Elution Analysis

II. Oligosaccharides

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During the last decade the study of chromatographic methods has made a most important contribution to analysis problems in sugar chemistry. A monograph [1] and several reviews have described the progress in this field [2, 3]. Different approaches have been made, using substituted or unsubstituted sugars, and both partition and adsorption methods have been applied. In adsorption systems both a polar adsorbent, as a clay [4], in a liquid less polar than water, and a nonpolar adsorbent (carbon) in water have been used. Especially for monosaccharides and the lower oligosaccharides both the qualitative separation and the quantitative analysis have been very successful.

For the higher oligosaccharides no good quantitative method has been published so far. Tiselius and Hahn [5] used displacement adsorption analysis for the resolution of a dextrinhydrolysate, but especially for the higher sugars the recovery was low due to irreversible adsorption. This was overcome by pretreating the carbon with a low concentration of the displacer. It was observed that by a stepwise increase in displacer concentration employed in the pretreatment, the sugars could be selectively washed from the column as elution peaks. Whistler and Durso have worked out a stepwise method, using etanol as an eluting agent [6], and Baily, Whelan and Peat have used this method for isolation of oligosaccharides with up to six glucose units [7]. A disadvantage of these methods is that the intermixing between adjacent components can be rather large and components present in small amounts can not easily be detected. Hough, Jones and Wadman mention [8] that they have applied paperchromatographic methods with success to the analysis of oligosaccharides

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without giving detailed results. Recently there has appeared a work by Jeanes, Wise and Dimler [9] describing a paper chromatographic method which is capable of resolving mixtures of oligosaccharides up to ten glucose units in a single molecule. No quantitative use of the method has yet been reported.

In the method for chromatographic analysis of oligosaccharides presented here a continuously increasing concentration of ethanol in water is applied to the saccharides which have been previously adsorbed on a carbon adsorbent. An earlier paper has discussed some of the general features of this technique [10], for which the name gradient elution analysis has been proposed.

On applying a concentration gradient of an eluting agent to a mixture of oligosaccharides adsorbed on carbon the higher members were only eluted with large losses of material, and as in displacement analysis this was overcome by pretreatment of the carbon. Such a pretreatment has been discussed earlier by other workers [11]. Thus Synge and Tiselius [12] used stearic acid in some model experiments with amino acids and peptides. The same compound has been tried with success in this work but employing a higher load. In addition to its advantageous effect on the adsorption properties of carbon a great convenience of the stearic acid pretreatment is that the compound is not dissolved from the carbon at moderate concentration of ethanol in water and so no contamination of the effluent occurs. The combination of pretreatment and a concentration gradient proved to be a most efficient method for the quantitative resolution of mixtures of oligosaccharides.

EXPERIMENTAL PART

Specimens of unhydrolysed and partially hydrolysed α-Schardingerdextrin were used as test substances. The author is grateful to Professor K. Myrbäck and Mrs. M. Jaarma Phil. Mag., Stockholms Högskola, for these samples. The dextrin is supposed to consist of a cyclic combination of six glucose units and the hydrolysate of it was considered to be a mixture of mono- to hexasaccharides and unchanged dextrin. (After some time it was observed that bacterial growth had developed in one of the samples.)

Three different carbons were tried as adsorbents. Carboraffin C, Norite FNX Special and Darco G-60. The carbons were usually mixed with an equal weight of Celite (analytical grade) and the pretreatment carried out by saturation in a column with the stearic acid solution in 96 % ethanol. The carbon was pressed out of the container and freed from adhering solution on a suction filter. It was stirred on the filter with 50 % ethanol, sucked dry and the operation repeated with 20 % ethanol. It was stored in glassbottles with 10 % ethanol. Negligible amounts of stearic acid were given off from the carbon by treatment with 50 % ethanol.

The adsorbent containers were made of Perspex. The carbon-Celite mixture was poured into the container in a thick slurry and allowed to settle under slight suction, afterwards it was pressed somewhat together with a stamper. After washing the column with water the surface was made as even as possible and sucked dry before applying

the solution to be analyzed, this was fed from an "Agla" micrometer syringe. The solution was sucked into the column under slight suction and followed by the same volume of distilled water, usually 0.3 ml. The empty space up to the top of the column, ca. 10 mm, was packed with moistened Celite which protected the adsorbent from being disturbed by the moving rod in the mixing chamber. The rate of flow through the column was kept at 8—10 ml/hour by applying an air pressure of ca. 0.2 atm.

The making of the gradient is described in detail in the previous paper [10] on gradient elution analysis. A "Shandon" automatic fraction collector was employed for collecting the fractions. It works on a weight basis and is equipped with weight-calibrated tubes. The smallest volume which could be used with some accuracy was 0.5 ml.

The orcinol-sulfuric acid reaction was used for the analysis of the fractions. The reaction is treated extensively by Sörensen and Haugaard [13], but the procedure suggested was somewhat modified in order to get a more rational method for the great number of fractions to be analyzed. The following reagents were required:

a) 15 g orcinol was dissolved in 500 ml water and added to a cooled mixture of 300 ml water and 200 ml conc. sulfuric acid.

b) 1800 ml conc. sulfuric acid was mixed with 1200 ml water and cooled.

In performing the analyses 60 ml of a) was mixed with 450 ml of b) and an aliquot of it was added to each tube with an automatic pipette, 8.0 ml of this mixture to each fraction of 0.5 ml. The volume in the tubes was too large to allow mixing by shaking and it was inconvenient to turn the tubes because the rim was apt to be contaminated with the strong sulfuric acid reagent. In order to overcome this difficulty a method of simultaneous mixing of the ingredients of all the tubes with glass rods was devised. Each series of measurements was accompanied through all operations by a set of control tubes containing known amounts of sugars (different dilutions of the solution applied to the column for analysis) which served as calibration points for the analytical procedure.

The tubes were heated for 15 min. in a boiling water bath, cooled down to room temperature in tap water and the extinction measured at 500 mμ in a Beckman spectrophotometer model B. This was equipped with an instalment for reading the tubes directly. The "Shandon" tubes were not fitted for immediate use in a spectrophotometer as they had heavy walls of somewhat uneven character. The simple devise of observing each tube through a complete rotation and then taking the smallest extinction as the real value was shown to be satisfactory. Tubes giving larger deviations from the mean spectrophotometer reading than $\pm$ 1.5 % by control with a solution of optical density 0.7 were not used.

The accuracy of the analytical procedure was limited by an absolute uncertainty in the spectrophotometer reading of about $\pm$ 0.01 corresponding to ca. 0.003 mg glucose and a relative error of about 5 %, the main sources of error being the uneven character of the tubes used and the variation in the fraction sizes which could amount to $\pm$ 10 %. This latter factor had an effect because the reaction was sensitive to the sulfuric acid concentration. The varying ethanol concentration in the fractions was shown to have only a very slight effect.

In the work of Sörensen and Haugaard [13] it is demonstrated that in lower oligosaccharides the monosaccharide unit gives the same analytical value as the monosaccharide itself, when correction is applied for the water taken up by hydrolysis. This was not given any further trial for the sugars analysed here and the analytical figures for the different peaks in the diagrams refer to amount of sugar calculated as glucose. The control solutions were made up from the solutions applied to the column.

The total work-time required for the chromatographic analysis of a sugar mixture using 100 fractions was about 5 hours.

RESULTS AND DISCUSSION

The general mode of operation of "gradient elution analysis" is treated in a previous paper [10] and will not be discussed here.

Five different preparations of α-Schardingerdextrin have been employed in the experiments reported below. They are designated here: 1) *Hydrolysate I*, which was contaminated by bacterial growth. 2) *Hydrolysate I, f 6*, a fraction obtained from Hydrolysate I by displacement adsorption analysis [5] and containing tetra- and penta-saccharides. 3) *Hydrolysate I, M*, a mixture of fractions from displacement analysis of Hydrolysate I containing reduced amounts of the lower sugars, 4) *Hydrolysate II*, obtained by hydrolysis of a highly purified dextrin. 5) *Unhydrolysed α-Schardingerdextrin.*

The hydrolysate is supposed to contain a mixture of mono- to hexa-saccharides and unchanged dextrine *i.e.* seven components and the chromatographic analysis shows the presence of these seven components. The α-Schardingerdextrin is the most strongly adsorbed as is seen by comparison of the experiments recorded in Fig. 2 H and I. So far no identification of the other peaks has been done but it seems clear that they leave the column in the order of increasing molecular weight [7].

Different degrees of saturation with stearic acid were tried in the pretreatment. The degree of saturation finally adopted was that obtained by equilibrating the adsorbent with 1 % stearic acid in 96 % ethanol. In Fig. 2 A and B is shown the effect of pretreating with a 0.2 % stearic acid solution (80 mg of stearic acid being adsorbed pr. g adsorbent). However, equilibrating with a 1 % solution (150 mg stearic acid adsorbed pr. g adsorbent) was better for the recovery of the highest sugars. With this pretreatment the carbon still had a high adsorption activity and separation power. Thus even the disaccharide had a curved isotherm in water as judged from Fig. 2 G, which shows that the elution peak of this saccharide is of an unsymmetrical form, corresponding to a curved isotherm under the condition of the elution, *i.e.* a very slight butanol gradient.

In the calculation of the recovery the large number of fractions should diminish the analytical error described above. On the other hand an exact determination of the baseline is difficult and small uncertainties in its position can cause slight errors in the recovery figures. Although the recovery cannot be rigidly controlled because of this uncertainty it is believed that there is substantially no loss of material during the passage of the sugars through the column.

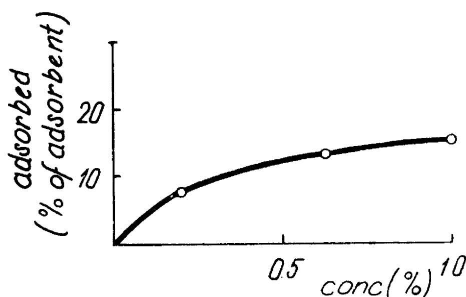

Fig. 1. Adsorption isotherm of stearic acid in 96 % ethanol on Darco G-60.

From the adsorption isotherm of stearic acid in 96 % ethanol of Darco G-60, shown in Fig. 2, it is seen that a concentration of 1 % of stearic acid gives a high degree of saturation of the carbon surface. One might expect that the stearic acid in addition to neutralizing the most active spots, modifies the properties of the whole surface. On the basis of the present experiments it is not possible to say what influence the pretreatment may have on the specificity although such influences might well be expected. The pretreatment of carbon should be compared with the deactivation of polar adsorbents by water as discussed for example by Stewart [14].

Later work in this laboratory has shown that the general effect of different saturators on carbon is much the same [15].

The stearic acid treatment caused depressed wetting properties of the carbon and flocculation of the carbon particles. This was to some degree counteracted by mixing the carbon with Celite and by adding ethanol to the adsorbent slurry before packing of the columns but clusters of flocculated particles must still be expected. This will have a bad effect on column efficiency and the spreading of zones. Pretreatment with a compound which produces, in addition to the deactivation characteristic of stearic acid, improved suspension properties of the carbons, should be expected to improve the column efficiency.

The pretreated carbon can be used repeatedly in chromatographic runs without noticeable changes in properties. The experiments recorded in Fig. 2 G and H are the 4th and 5th respectively carried out on the same column. Between the runs the column was washed with 33 % ethanol and water.

Other eluting agents than ethanol were tried. Propanol and metyletylketone gave very similar results. Their eluting power relative to ethanol was ca. 2 and 5 respectively. Normal butanol was too little soluble in water to be usable as is seen from the experiment recorded in Fig. 2 G, where a nearly saturated n-butanol solution in water is fed to the mixing chamber. The approximately straight lines drawn in Fig. 2 G and I denote the concentration in effluent of butanol and ethanol respectively calculated as described [10]. The

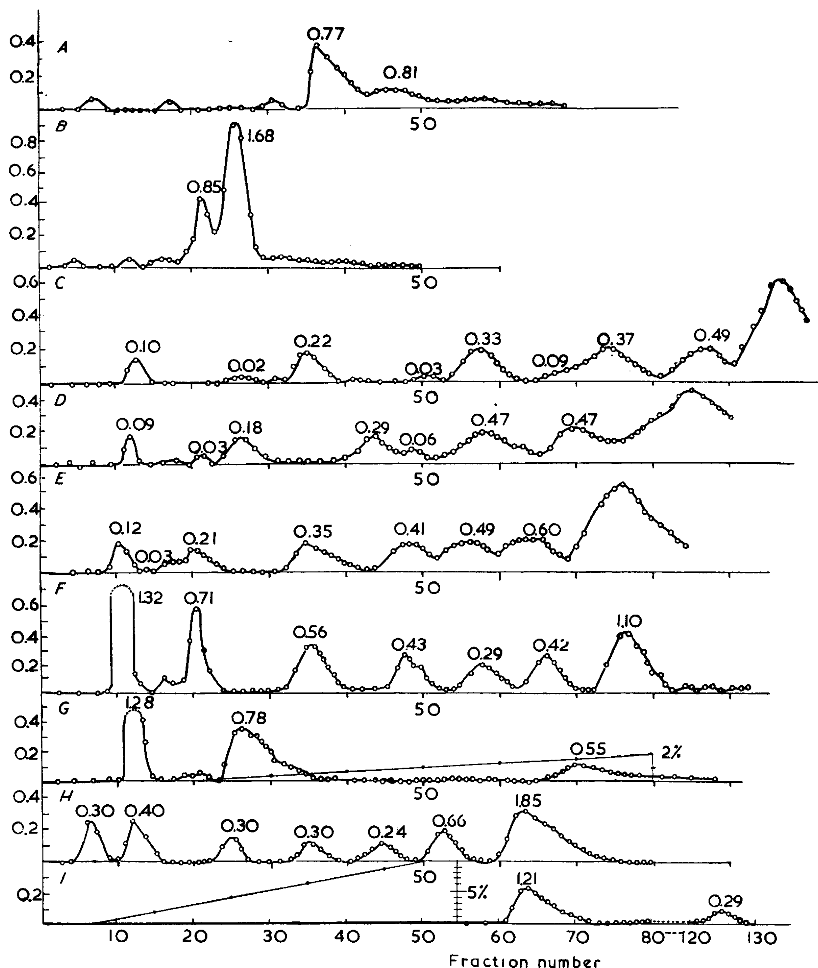

Fig. 2. Adsorbent: A, B, E, F, G, H, I, Darco G-60 + Celite 1/1: C, Carboraffin C + Celite 1/1; D, Norite FNX Special + Celite 1/1.

Column size: A, B, 8 × 60 mm; C, D, E, F, G, 8 × 130 mm; H, I, 10 × 200 mm.

Pretreatment: A, none; B, equilibrated with 0.2 % stearic acid in 96 % ethanol; C, D, E, F, G, H, I, equilibrated with 1 % stearic acid in 96 % ethanol.

Substance analysed: A, 3.0 mg, B 3.2 mg. Hydrolysate I f 6; C, D, E, 5.0 mg. Hydrolysate I M; F, G, 5.0 mg Hydrolysate I; H, 4.20 mg. Hydrolysate II; I, 1.50 mg. Unhydrolysed-a-Schardingerdextrin.

Volume of mixing chamber: A, B, C, D, E, F, G, 62 ml.; H, I, 256 ml.

Solution fed to mixing chamber: A, B, 50 % ethanol; C, D, E, F, H, I, 30 % ethanol; G 5.6 % (by weight) n-butanol. In I changed to 96 % from the 98th fraction. Fraction size: A, B, C, D, E, F, G, 0.50 ml.; H, I, 2.1 ml.

Recovery: A 57 %; B 94 %; F 98 %; H 97 %; I 100 %.

The dead volume is equivalent to the retention volume of the front of the monosaccharide peak. The amount of sugar is designated by a number (mgs.) above each peak.

butanol values are corrected for the observed retention volume in addition to the dead volume of the column. The circles denote analytical values found by examining the effluent with an Abbe refractometer. It is seen that ethanol has a negligible retention volume on the pretreated carbon, while butanol, though it has a noticeable retention volume, must have a linear adsorption isotherm in concentrations up to 2 %.

A component travelling in an eluting agent of increasing concentration will move through the column with an increasing speed [10], and the volume of solvent in which it leaves the column is dependent upon the mutual relationship, betwen the gradient slope and column size. The conditions are usually so chosen that the sugars will leave the column when travelling with low adsorption and consequently in a small volume. The importance of this point for the quantitative estimation of the component is seen by a comparison of the trisaccharide peak in Fig. 2 F with that in Fig. 2 G.

The three different carbons employed showed a marked difference in specificity with regard to the α-Schardingerdextrin as is seen from the relative positions of the dextrin peak in Fig. 2, C,D,E. When Darco G-60 is used, the dextrin appears after and separated from the hexasaccharide while on Carboraffin C it appears close to and partially mixed with the pentasaccharide. Norite FNX Special has an intermediate effect. *Hydrolysate I*, which was contaminated by bacterial growth contains some additional compounds in small amounts. The full resolution of these trace components has not been attempted, and they are probably to some degree the cause of the "tailing" exhibited by some of the peaks in Fig. 2 F being partially mixed with the main components.

In Fig. 2 H and I are shown the results of analyses of *Hydrolysate II* and *Unhydrolysed α-Schardingerdextrin*. The second peak in Fig. 2 I is not identified, but is probably the β-Schardingerdextrin. It should be noted that the solution fed to the mixing chamber is changed to 96 % ethanol after the 97 th fraction. The fractions 81—119 which are not plotted in the diagram, contained no sugars. (Because of the large fraction sixe in these runs aliquotes were pipetted out for the quantitative analysis.)

It seems that the application of "gradient elution analysis" in combination with pretreatment of the carbon adsorbents offers great possibilities for the quantitative resolution of sugar mixtures including those containing much larger molecules than present in the α-Schardingerdextrinhydrolysate. The work described here obviously can be subject to several refinements in technique such as an improvement in column efficiency and a more accurate quantitative analysis of the fractions.

SUMMARY

A new method for the quantitative chromatographic analysis of mixtures of oligosaccharides is described.

The method is characterized by the application of a continuously increasing concentration of ethanol in water, as an eluting agent, applied to a mixture of sugars adsorbed on a carbon adsorbent which has been pretreated with stearic acid.

The analysis of a hydrolysate of α-Schardingerdextrin containing a mixture of mono- to hexa-saccharides and unchanged dextrin is described.

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