AN ANALYTICAL METHOD FOR OLIGOSACCHARIDES IN SUGARCANE PRODUCTS

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SUMMARY

An analytical method has been developed which can be used for routine detection and estimation of oligosaccharides in cane sugar products. Levels as low as 0.01% can be detected and oligosaccharides have been found in all mill and refinery products.

The method is based on gradient elution with aqueous ethanol, using a carbon-celite column. Samples of about 200 mg give good results for most products. About five hours are needed for the complete analysis because preliminary elutions necessary to remove the bulk of the sucrose before the gradient is applied.

The oligosaccharides usually present in cane products can be resolved into three major components and their relative proportions can be estimated. The degree of crystal growth inhibition which might be expected can be assessed from the presence of 1-kestose, neo-kestose and another oligosaccharide (aGL-1); all oligosaccharides contribute to the optical rotation measured for pol determinations. The other minor and trace compounds cannot be easily distinguished as individual components.

INTRODUCTION

A comprehensive study of sucrose crystal growth has been in progress in these laboratories over the past nine years. Recent publication of some of this work by Smyth1,2,3 has shown that two main steps, mass transfer to the crystal surface and the incorporation of the sucrose molecule into the growing crystal, are important in sucrose crystal growth kinetics.

From this work has arisen the concept of two broad classes of impurities—those that retard crystal growth (at constant supersaturation) by hindering the rate of mass transfer and those that inhibit the incorporation of sucrose into the crystal lattice by poisoning the growing crystal surfaces by adsorption. While the majority of impurities found in natural sugar syrups affect crystal growth rates mainly through hindering mass transfer, a particular class of materials has been clearly implicated as sucrose crystal growth poisons. These poisons belong to the class of sugars described as oligosaccharides. Not all the inhibiting oligosaccharides have been fully characterised as yet but they have in common a sucrose moiety in which an additional monosaccharide substituent is attached to a primary hydroxyl group of sucrose. The substituents may be glucose, fructose or galactose; the addition of further monosaccharide units on the original substituent only slightly decreases the poisoning effect. The most powerful of these inhibiting oligosaccharides are those in which the substituent is on the primary hydroxyl group of carbon atom 6 in the glucose moiety of sucrose.
Oligosaccharides not only inhibit the rate of growth of sucrose crystals, but lead to marked changes in the shape of the crystals due to their selective adsorption on different faces of the sugar crystal. Each inhibitor has a specific mode of adsorption and the crystal shape and reduction in growth rate can be estimated from a knowledge of both the oligosaccharides present and their concentrations.

Work by Gagolski, which is continuing in these laboratories, has led to the separation and identification of a number of pure oligosaccharides from various cane products. These sugars are all optically active with widely varying specific rotations. The usual mixture found in sugarcane juices gives a rotation approximately equivalent to an equal weight of sucrose. This suggests that oligosaccharide levels are of interest in their own right due to their adverse effects on the accuracy of standard polarization methods for the determination of sucrose.

This present work has been aimed at measuring the overall levels of oligosaccharides in cane-derived materials, with emphasis on the distinction between powerful inhibitors of crystal growth and those which have a lesser effect.

METHOD OF ANALYSIS

The usual methods for the identification and estimation of oligosaccharides are based on paper chromatography and the selection of appropriate solvent systems. The other alternative is to carry out a normal preparative separation of the compounds by column chromatography, using the precautions necessary for quantitative work. The first scheme is subject to the vagaries of the sensitivity of reagents to the various sugars and the difficulty of obtaining good separations with an impure system. The scale of operation of the second method is not satisfactory for routine work.

Our method is based on column chromatography for the routine analysis and paper chromatography was used to check the method. The method with most promise of speed and reasonable separation was gradient elution from a small column. Gagolski had successfully used large columns for preparative work; his technique using a carbon-celite stationary phase and aqueous ethanol as eluant was adopted.

Equal weights of Celite 545 and Darco Carbon G-60 are mixed with water as a dilute slurry, filtered on sintered glass, and dried at 90°C under vacuum. The dried carbon is allowed to age for a day in air before use.

Long columns should give the best separation for a fixed carbon charge. Columns with length to diameter ratios (L/D) of 5 to 20 were tested, but the short columns showed no loss of resolution.

A standard column size equivalent to 4.5 g carbon-celite was selected. The dimensions are:

- internal diam. 14.5 mm
- total length 120 mm
- bed length 80 mm
- L/D 5.5

The bed is supported by a sintered glass disc with a thin (1-2 mm) bed of Celite 535 to prevent fouling of the disc.

A column is prepared by mixing 0.05 g Celite 535 with 5 ml of water, pouring into the glass column, and allowing to drain. Then 4.5 g premixed carbon plus
15.5 g water are mixed and poured into the column. After some settling and removing any greasy carbon scum, 3% aqueous ethanol is pumped at 3 ml/min for 10 minutes to consolidate the carbon bed. The sample is added to the column in the usual way and followed with rinse water. The primary elution can then begin.

Sample volumes are generally about 1 ml at 20 brix, containing approximately 200 mg sucrose. This is suitable for oligosaccharide determinations on most cane-based samples. Weights up to 1000 mg may be used on refined and raw sugars, while 50 or 100 mg samples may be adequate for molasses and closely related materials.

Considering the large excess of sucrose over oligosaccharides, a preliminary elution step is necessary to elute the sucrose before a gradient elution is used to separate the various oligosaccharides. However, a clean cut cannot be made between sucrose and the other oligosaccharides since the physico-chemical properties which contribute to their separation on a carbon column show no such sharp differences. Disaccharides are either eluted before the sucrose, with the main bulk, or in the sucrose tail. Some trisaccharides are eluted by the low concentrations of ethanol which are suitable for the preliminary elution of sucrose. Fortunately we have found that those compounds removed during the sucrose elution are neither potent inhibitors nor present in significant quantities. Thus, the loss of these minor components does not invalidate the method.

The preliminary elution is made with 500 ml of 3% (by volume) aqueous ethanol, pumped at 3-3.5 ml/min. This eluate is discarded. Faster elution rates reduce the total amount of sucrose rejected. The main eluant is made from 120 ml of 3% alcohol contained in a stirred beaker (see Fig. 1) and 120 ml of 30% alcohol in a second beaker, connected to the first by a glass tube between the bases. As eluant is pumped from the first beaker, the flow from the second causes the ethanol concentration to increase almost linearly. The volume of gradient pumped is 155 ml at 1.4 ml/min.

The preliminary trials were evaluated by paper chromatography, collecting the eluate from the column in 1 or 2-ml fractions before spotting. However, a colori-
metric method offered more rapid results once the separational techniques had been refined sufficiently to differentiate between the various oligosaccharides.

The anthrone method, which is specific for hexose sugars, was chosen. The column eluate is mixed with anthrone dissolved in 76% sulphuric acid. The oligosaccharides are hydrolysed to hexoses that react with anthrone to give a blue colour, which is read at 625 mp. This technique was tried manually and gives good results but is not suitable for routine work.

An Auto-Analyser was commissioned, using a modification of the manual anthrone method. The sample from the column is split, pumping 1.0 ml/min (all volumes are nominal values for Auto-Analyser pump tubing) for analysis and rejecting the remainder. The sample is mixed with 0.8 ml/min air and 2.8 ml/min anthrone reagent (80% sulphuric acid by volume plus 1 g anthrone per litre) and then heated to 95°C for 6 min in an oil bath. The percent transmission after colour development is measured by the colorimeter and recorded as a function of time by the recorder (see Figs. 2–7). In fact, the time axis of the graph is an elution volume scale.

![Image of elution volume graph](image)

**Fig. 2.** Mixture of oligosaccharides (non-standard conditions—normal gradient elution, no preliminary elution). A = levanose, B = 1-levanose, C = 0/51, D = 0/3, E = neo-levanose, F = 0/5.

The data recorded on the chart correspond to the period during which the proportioning pump is taking liquid from the gradient mixer. Thus, the graph shows the residual sucrose which was already being eluted from the column when the gradient commenced, as well as the oligosaccharides eluted by the gradient. All records are the same in this respect; the starting point is the time of connection of pump to gradient.

Conversion of the graphical results to optical density or absorbancy values enables a quantitative assessment of the total amount of oligosaccharides to be made. The different oligosaccharides show somewhat different sensitivities to anthrone but the variation is small and an average value for the various oligosaccharides present in cane samples has been taken.

* * Technicon Pty. Ltd.
The graph also gives data in the form of elution volumes, and this can be used to identify the various oligosaccharides. The elution volume under fixed conditions is a diagnostic property of the various oligosaccharides, but the large number of compounds isolated to date make it difficult to use as a sole means of identification. Although Gagolski has isolated some twenty different oligosaccharides, only five occur regularly and in significant amounts. Further, only three are responsible for about 80–90% of the total oligosaccharide level in typical samples. Because of these considerations, the elution volumes can be used to identify major components with reasonable confidence.
The system calibration falls into three parts. Firstly, the elution volumes of various oligosaccharides have been measured. Both commercially available materials and compounds isolated from cane products were used. Secondly, the response to anthrone of a fixed amount of oligosaccharide placed on the column and eluted in the standard method was determined. There is no sign of a significant permanent loss of material by irreversible absorption on the carbon. Lastly, the day-to-day sensitivity of the system is checked each day by using a sucrose solution of standard strength as input to the sample line.

RESULTS

It is convenient to express elution volumes in terms of the distance along the chart from the starting point. Since standard pumping rates and charts have been used, all such comparisons are valid; the chart distance is quoted in the values below.

A number of oligosaccharides are listed below and elution values given. Values are reliable to ± 1 and are given only in steps of 1/4.

The table, which does not include all the oligosaccharides so far isolated from cane products, shows that one compound (21/3C) is eluted during the preliminary elution—although some still remains. There is at least one (26/2B) which is eluted between 4½–6½ which cannot be easily detected because of the large peaks due to residual sucrose (4½) and 1-kestose.

A comparison of Figs. 2 and 3 shows that the preliminary elution reduces the sharpness of the peaks and alters some elution volumes, but allows 1-kestose and
TABLE 1
RELATIVE STANDARDIZED ELUTION VALUES

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Elution value</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raffinose</td>
<td>8</td>
<td>not found in cane; inhibitor</td>
</tr>
<tr>
<td>Stachyose</td>
<td>9</td>
<td>not found in cane: inhibitor</td>
</tr>
<tr>
<td>Melezitose</td>
<td>6</td>
<td>not found in cane</td>
</tr>
<tr>
<td>Kestose</td>
<td>4</td>
<td>not found in cane; inhibitor</td>
</tr>
<tr>
<td>16/2B*</td>
<td>5½</td>
<td>trace</td>
</tr>
<tr>
<td>21/3C*</td>
<td>eluted early</td>
<td>trace</td>
</tr>
<tr>
<td>1-kestose</td>
<td>6</td>
<td>major</td>
</tr>
<tr>
<td>neo-kestose</td>
<td>8</td>
<td>major; inhibitor</td>
</tr>
<tr>
<td>0/3</td>
<td>8</td>
<td>minor</td>
</tr>
<tr>
<td>0/5</td>
<td>11</td>
<td>minor; inhibitor</td>
</tr>
<tr>
<td>0/5-1</td>
<td>7</td>
<td>major; inhibitor</td>
</tr>
</tbody>
</table>

* Numbers indicate pure oligosaccharides isolated from various fractions of cane-derived materials. Their precise structure will be reported in a later publication.

0/5-1 to be separated. The preliminary elution has obviously caused differential movement of oligosaccharides down the column and allowed a vital separation to be made. Neo-kestose and 0/3 are not resolved but paper chromatography shows that 0/3 is generally only a minor component.

Residual sucrose always gives a definite peak at about 4½ and its presence makes the detection of minor components difficult. The influence of the residual sucrose can be practically eliminated if 750-1000 ml of 3% aqueous ethanol is used for the preliminary elution. However, this considerably lengthens the total time required, badly distorts the 1-kestose peak, and causes more complete elution of the minor materials before 1-kestose that are only partially removed with a 500 ml elution.

An allowance can be made for residual sucrose, based on the height of the graph between about 1 and 3; standard curves are prepared using double-recrystallized sugar. The method is able to detect oligosaccharide levels as low as 0.01% in 200 mg of sucrose, i.e. equivalent to 0.02 mg. At these levels there is a considerable degree of uncertainty in the estimation but the detection is unequivocal. Levels as high as 2% can be measured in 200 mg of sample (i.e. 4 mg oligosaccharide) although it is preferable to reduce the sample size to 50-100 mg. Larger samples up to 1000 mg, can be handled successfully when low oligosaccharide levels are suspected.

Some typical analyses are given in Table 2 but the figures represent only a small range of samples (e.g. only one refined sugar). It can be seen that oligosac-

TABLE 2
ANALYTICAL RESULTS

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number tested</th>
<th>Oligosaccharides % solids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refined Sugar</td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td>Raw Sugars</td>
<td>4</td>
<td>0.02-0.2</td>
</tr>
<tr>
<td>Cane Juices</td>
<td>12</td>
<td>0.08-0.5</td>
</tr>
<tr>
<td>C Massecuites (Mill)</td>
<td>4</td>
<td>0.8-1.2</td>
</tr>
<tr>
<td>CBO Massecuites (Refinery)</td>
<td>4</td>
<td>1.5-2.7</td>
</tr>
<tr>
<td>CBO Syrups</td>
<td>5</td>
<td>2.5-3.5</td>
</tr>
</tbody>
</table>
Oligosaccharides are present at all stages of milling and refining and that the percentage in a particular product can vary over a wide range.

Although only 30 samples are quoted above, a large number of other samples (e.g., BBO syrups, liquors) have been examined and a mill trial has been instituted, which will involve hundreds of analyses.

Reference should also be made to Figs. 4–7. These demonstrate the typical results which are obtained. For instance, the refinery CBO syrup (molasses) clearly contains observable amounts of five oligosaccharides—as well as a considerable number of trace constituents. The major component is 1-kestose, with large amounts of 0/5-1 and neo-kestose, a small amount of 0/5 and an uncharacterized substance marked E. We can also assume the presence of 0/3; paper chromatography confirmed this assumption.

The cane juice contains significant quantities of 1-kestose, 0/5-1 and neo-kestose in proportions that are typical of most mill products. The raw sugar is interesting in that the major components are now 0/5-1 and neo-kestose. Other raw sugars have previously been found to contain approximately equal amounts of the three main oligosaccharides present in cane juice.

DISCUSSION

The analyses have shown the presence of oligosaccharides in all cane sugar products. Analytical grades have been found to contain observable amounts (about 0.01%) and only twice recrystallized material can be regarded as free of detectable oligosaccharides. This method has also been used to demonstrate the known presence of raffinose in raw and refined beet sugar.

The analytical procedure itself has been considerably streamlined since it was first used and a two-pen recorder is now in use with two colorimeters, a single heating bath, and a single pump. The system handles 14 analyses per day on a two shift basis, allowing time for calibration checks.

The sample preparation and preliminary elution takes about 3 h, while the main gradient elution takes 110 minutes. Thus, the Auto-Analyser is occupied for 2 hours by each sample, allowing time for rinsing between samples and base-line correction.

We have found that activated carbons are subject to considerable variability in properties. Five different carbons tested showed quite different resolving powers and it was also necessary to check the performance of a new batch of a known grade of carbon. Slight changes in process specifications for a fixed grade of carbon can cause a marked change in chromatographic properties. However, the carbon properties do not seem to be affected by long-term storage.

The most interesting effect observed in this work has been the considerable degree of concentration of the inhibiting oligosaccharides during the crystallization stage. Their surface adsorption during crystal growth leads to a degree of permanent inclusion in the final crystal. Because of this effect, refinery boil-out syrups contain much higher relative proportions of 0/5-1 and neo-kestose than the equivalent mill syrups. The syrup layer on raw sugar contains a selection of all oligosaccharides and serves to transport 1-kestose to the refinery.
ACKNOWLEDGEMENTS

Appreciation is expressed to the Management of the Colonial Sugar Refining Company Ltd. for permission to publish this paper.

The assistance of Mrs. J. Steele and Mrs. E. Nicholls in the routine laboratory work is gratefully recognized.

REFERENCES

1 GAGOLSKI, J. Unpublished data.

Discussion

MATIC: In view of considerable overlapping of peaks during separation, how accurate are the percentage figures quoted?

STAIZER: The accuracy of the percentages is admittedly limited; however this is better than any prior information. At the present stage of development of the method, the percentages are gross measurements and individual problems can only be approximately assessed.

MATIC: Have you measured oligosaccharides in final molasses?

STAIZER: Yes, please refer to the final molasses data as reported in the paper.

TSANG LEUNG YIN: What does “CBO” stand for in the last table just shown?

STAIZER: This is merely local jargon referring to the final boil out product of a refinery or “C” Boil Out—commonly final molasses in a refinery.