THE ISOLATION AND PARTIAL CHARACTERISATION OF OLIGOSACCHARIDES IN REFINERY MOLASSES

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Abstract

Two trisaccharides have been isolated by carbon chromatography from cane refinery molasses. Raffinose was detected and characterised by hydrolysis. The other carbohydrate yielded, on hydrolysis, fructose and glucose in the ratio of 2:1, indicating a fructosylsucrose. Concentrations of each trisaccharide were approx. 0.5% by weight.

Introduction

The determination of sucrose in molasses is of prime importance to the efficient running of a sugarcane factory. When the conventional methods for sucrose determination were drawn up, the influence of impurities such as raffinose, kestoses and polysaccharides was not fully realised.

The application of the invertase method in beet molasses was questionable because of the presence of raffinose, kestose and levan[39]. These compounds are all hydrolysed to monosaccharides and thus a falsely high sucrose value is obtained. The polarisation methods for the determination of sucrose are also suspect because of oligosaccharide interference[39,10]. De Whalley[13] has noted that a raw sugar shows an elevation of pol of 0.4 for every 1% kestose present. Clerget molasses, however, shows an increase in sucrose content of 0.8 for the same concentration of kestose present.

Sutherland[40], along with Leonard and Richards[30], identified polysaccharides as being the major cause of crystal elongation. On the other hand, however, Japanese workers [28], concerned about the refining quality of raw sugars, have considered the effects of starch, silica, phosphate, gum and oligosaccharide impurities on the formation of needle-shaped crystals in raw sugars. They concluded that oligosaccharides are a dominant factor and gums a secondary factor in the formation of elongated crystals. Extensive studies into the kinetics of sucrose crystal growth have been carried out by Smythe[36,37,38]. Oligosaccharides have been found to poison the growing sucrose crystal by adsorption. The most effective inhibitors of sucrose crystal growth appear to be those oligosaccharides derived from sucrose by substitution on the primary hydroxyl group attached to C_6 of glucose. Therefore raffinose and neo-kestose (II) are reported to be much more powerful inhibitors than 1-kestose (III) and 6-kestose (IV).

These oligosaccharides have the same sucrose moiety and their occurrence is due to transglycosylation. Kestoses are fructosylsucroses which have been formed by the enzymatic transfer of a fructose radical to one of three primary hydroxyl groups of sucrose by the action of invertase[17]. The formation of these fructosylsucroses has been observed with invertase from a wide variety of sources—fungal, bacterial and plant extracts[1,3,4,5,7,22,25]. Methods of preparation of these kestoses have been outlined by Gross[21]. One of these methods was modified slightly and used to prepare 6-kestose in our laboratories.

Raffinose, although occurring mainly in beet sugar factories, has been reported in cane sugar products. Gross[23] detected raffinose in raw sugar produced from cane. Binkley[9] later verified this by isolating raffinose and crystalline 1- and 6-kestose[8] from cane final molasses.

It was decided therefore to study the occurrence of oligosaccharides in South African sugar factories. Refinery molasses was chosen as the initial starting material.

Several chromatographic media have been used for the separation of sugars. Ion exchange[32] and more recently, dextran[18] and polyacrylamide gels[27,34] have been used in the separation of carbohydrates.

Carbon was first proposed as a chromatographic medium for the separation of carbohydrates in 1941[41]. Six years later the separation of a mixture of glucose, sucrose and raffinose was reported using carbon as the stationary phase and a 4% aqueous phenol solution as eluant[11]. Whistler and Durso[43] extended the technique of Tiselius and in 1950 reported the use of aqueous ethanol as an eluting agent for sugars from a carbon-celite column. Small variations of eluant composition, degree of dilution of sugars and the presence of inorganic salts did not affect the separation.

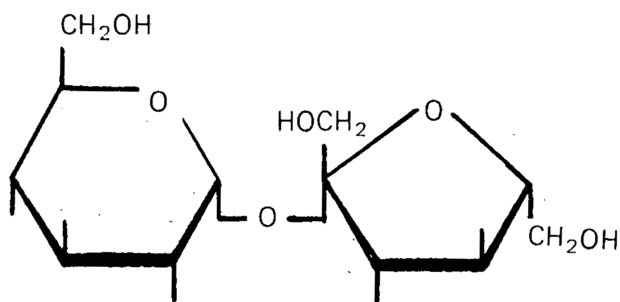
The method of Whistler and Durso[43] was adopted and proved successful in the isolation of two trisaccharides from refinery molasses.

Experimental

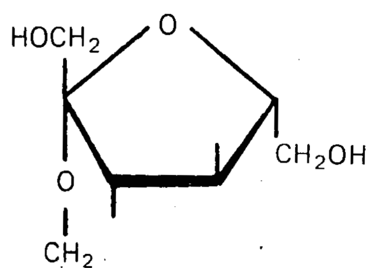
All concentrations were carried out at 40°C under reduced pressure.

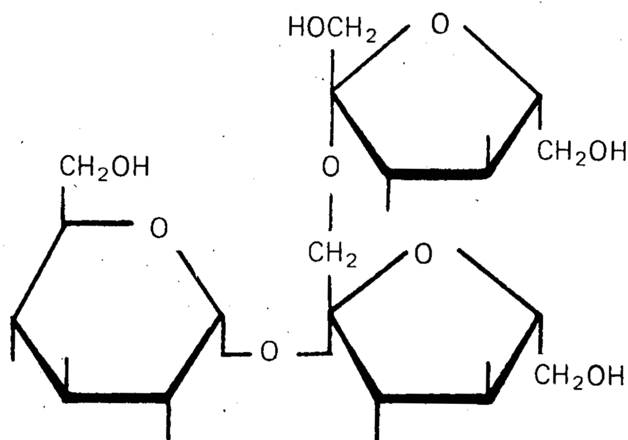
Paper Chromatography

Paper chromatography was carried out using the continuous descending technique on Whatman No. 1 paper, or (for preparative work) Whatman No. 3MM. The following solvent systems were used.



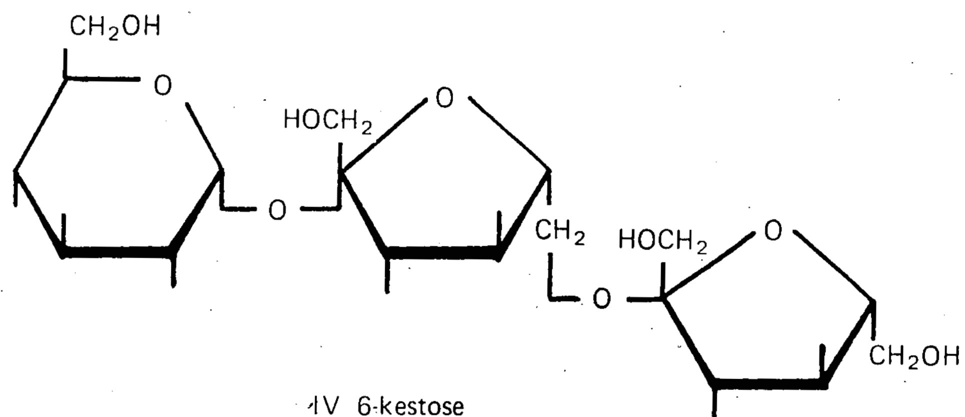
I. Sucrose





II Neo-kestose

III I-kestose



IV 6-kestose

A:[44] Butan-1-ol: Ethanol : water (2:1:1 by volume)
B:[2] Propan-1-ol: Ethylacetate : water (7:1:2 by volume)
C:[24] Propan-2-ol: Butan-2-one : Formdimethylamide : water (10:5:1:4 by volume)
D:[33] Butan-1-ol: Ethanol : water (4:1:5 by volume) upper phase.

Drying after solvent development was achieved at 100°C except in preparative work where drying was by a cold air draught.

Aniline-diphenylamine[6] was used as the detecting reagent.

Thin Layer Chromatography

Thin layer chromatography was performed on 20 x 20 cm plates using Silica Gel G (Merck, acc. to Stahl). The Silica Gel was slurried with water (unless otherwise stated), spread to a thickness of 0.35 mm, air-dried for 10 minutes and finally activated at 100°C for one hour.

The following solvent systems were used:

E: Ethylacetate : Acetic Acid : water (6:3:2 by volume)
F:[12] Chloroform : Acetic Acid : water (6:7:1 by volume)

Drying after solvent development was achieved at 100°C except where otherwise stated.

Aniline-diphenylamine[6] was used as the detecting reagent.

Characterisation of Carbon-Celite Column

The amylose sample was prepared by fractionation of potato starch according to the method of Gilbert[19]. Approximately 8 g of potato starch (B.D.H.) was used to give 0.6 g of amylose in the form of the stable butanol-amylose complex.

The amylose was partially hydrolysed using sulphuric acid[42]. The Somogyi-Nelson[26,31] micromethod for reducing sugars was used to follow the course of the hydrolysis. The reaction was stopped after 32.5% apparent conversion to glucose. Kuhn's[29] formula indicates that an optimum yield of dextrins containing from 3 to 8 glucose units would be present at this stage. The solution was de-ionised (Amberlite M.B.-1) and concentrated to 5 ml.

50 g of a carbon (B.D.H. acid washed) - celite (Hyflo Supercel) mixture (1:1) was slurried with water and packed into a column (350 x 25 mm). The column was compacted by passing through 500 ml of water at 3.0 ml/min. The hydrolysate (2 ml) was applied to the column and eluted with water at a flow rate of 1.5 ml/min. The eluant was collected in 15 ml fractions and monitored with anthrone[16]. A large carbohydrate peak was detected. After complete removal of this peak 5% EtOH was pumped through the column and the eluant again monitored with anthrone. The elution procedure was repeated stepwise to a final concentration of 30% EtOH.

The fractions containing the individual peaks were concentrated and spotted on Whatman No. 1 paper. The chromatogram was developed using Solvent A. After development (72 hours) the paper was dried, sprayed and the respective r_g values determined (Table II). Separation up to D.P. 10 with 30% EtOH confirmed that the entire oligosaccharide range had been covered.

TABLE II
$R_{glucose}$ values of fractionated amylose hydrolysate on Whatman No. 1 using solvent A

Fraction	$R_{glucose}$	$R_{glucose}$ of Hydrolysate
H_2O	1.00	1.00
5% EtOH	0.65	0.67
10% EtOH	0.42	0.40
15% EtOH	0.25	0.26
20% EtOH	0.17	0.18
	0.10	0.09
25% EtOH	0.07	0.05
	0.03	0.03
30% EtOH	0.02	unresolved

Isolation of the Oligosaccharides from Refinery Molasses

Initial Separation

A column (500 x 40 mm) was packed with a slurry of 270 g of a carbon (BDH acid washed) -celite (Hyflo Supercel) mixture (1:1) in water. The column was allowed to settle and compacted by pumping through 500 ml of 5% EtOH at a flow rate of 3.0 ml/min. Refinery molasses (5 g) was taken up in 20 ml of 5% EtOH, applied to the column, which was developed with 5% EtOH (2.0 ml/min.). The eluant was monitored with anthrone[16] and the fraction 840-3 000 ml on analysis by paper and T.L.C. showed the presence of glucose, fructose and sucrose only. A portion of this fraction was concentrated, hydrolysed with invertase and re-chromatographed. No disaccharides were detected.

The developing solvent was changed to 10% EtOH and collected in 15 ml fractions. The trisaccharide fraction was detected after 860 ml and was completely removed by 1 260 ml. Analysis showed two peaks incompletely separated. The fractions containing the trisaccharides were concentrated to 10 ml.

Purification of the 10% Fraction

The concentrated trisaccharides were re-chromatographed on a smaller column (350 × 25 mm) using 50 g of carbon-celite. The column was packed as in the amylose calibration. Sucrose was eluted with 5% EtOH and the trisaccharides were eluted with 10% EtOH at 1.5 ml/min. Two peaks, incompletely separated, were evident after analysis with anthrone (Fig. 1).

Semi-quantitative analysis with anthrone indicated 26 mg and 36 mg carbohydrate (as raffinose) in each peak (A and B respectively). The fractions containing the peaks were concentrated separately and analysed by paper chromatography (Solvent A).

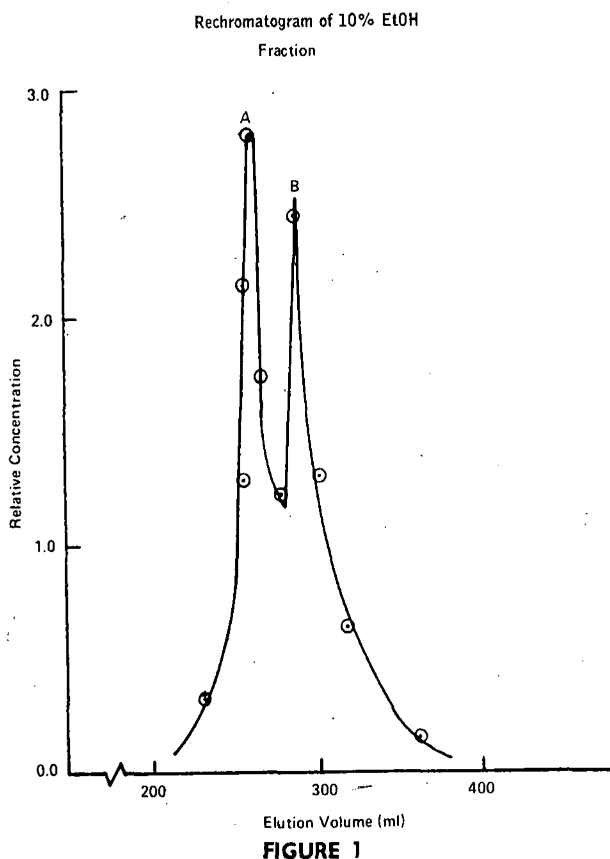


FIGURE 1

The concentrates from peaks A and B were applied to Whatman 3MM paper and developed with Solvent A for 48 hours. The papers were dried in a stream of cold air and indicator strips on either side of the paper sprayed. The sections corresponding to the desired carbohydrate spots (i.e. pure peak A or B) were cut from the unsprayed portion of the paper and the sugars eluted with water using the method of Dimler[14]. Elution was continued for two to three hours in a humidified cabinet during which time 8 to 12 drops of eluant containing all the carbohydrate was collected.

Hydrolysis

Partial acid hydrolyses were conducted with 0.005N H_2SO_4 for one hour at 60°C. Complete hydrolysis was effected with 0.05N H_2SO_4 for two hours at 100°C. Enzymatic hydrolysis was carried out by incubation for two hours at 60°C with invertase (10 mg Convertit/ml).

All hydrolyses were carried out using the ultramicrotechnique of Porter[35]. Using this method from 0.01 to 0.05 ml of sample containing about 20 to 50 μg of sugar was hydrolysed in sealed tubes approximately 100 × 2 mm. Acid solutions were neutralised with ion-exchange resin (Amberlite MB1) prior to paper chromatography (Solvent D).

T.L.C. of the peak B hydrolysate was carried out on Silica Gel G impregnated with sodium acetate buffer[12]. The plates were run three times using solvent F. Drying was effected in a stream of cold air between each run.

Fructose/Glucose Ratio of Peak A

The invert hydrolysate of pure peak A was analysed for total reducing sugars according to the Somogyi-Nelson method[26, 31] and for ketose using the resorcinol-HCl method[15, 20]. A fructose/glucose ratio of 1.7 : 1 was obtained.

Preparation of 6-kestose

6-kestose was prepared by a method based on that of Gross[31]. 150 gm of cellulose (Whatman CF 11) was slurried with the solvent isopropanol: butanol : water (7 : 1 : 2) and packed into a column (550 × 40 mm). After settling and compacting by pumping 300 ml of the same solvent through the column at 1.0 ml/min, 5 gm of the sucrose-invertase syrup[21] was taken up in the solvent and applied to the column. The solvent was pumped at a flow rate of 0.64 ml/min through the column and 6.4 ml fractions collected. Individual fractions were analysed by T.L.C. using solvent E. Crystallisation from absolute methanol was effected in a dessicator containing anhydrous silica gel m.p. 138-139° (lit., m.p. 144-145°).

Results and Discussion

Fractionation of the amylose hydrolysate on the carbon column yielded glucose, maltose, maltotriose and maltotetraose when eluted with water, 5% EtOH, 10% EtOH and 15% EtOH respectively. Similar analysis on a refinery molasses sample showed that no sugar was present in the 15% fraction and hence it was assumed that the two sugars eluted in the 10% peak were both trisaccharides. The EtOH concentration in the molasses fractionation was increased stepwise as shown in Fig. 2. It can be seen that the concentration of higher sugars is small compared with that of the trisaccharide fraction. From the amylose hydrolysate calibration the entire range of D.P. 1-10 was covered by increasing the EtOH concentration from 0 to 30%.

Analysis of peak A yielded the following results:—

1. Paper chromatography in several solvents gave $R_{sucrose}$ values characteristic of 1 or neokestose (Table I).
2. Invertase hydrolysis yielded glucose and fructose on analysis by chromatography. The linkages therefore must be of the β-type.
3. Complete acid hydrolysis gave glucose and fructose while partial hydrolysis yielded fructose, glucose, sucrose and the original sugar.
4. Determination of the fructose/glucose ratio after invert hydrolysis yielded a ratio of 1.7 : 1.

On the basis of these observations peak A has been tentatively classified as a kestose. Comparison of $R_{sucrose}$ values rules out the possibility of 6-kestose. Electrophoretic analysis will show whether 1- and/or neo-kestose is present.

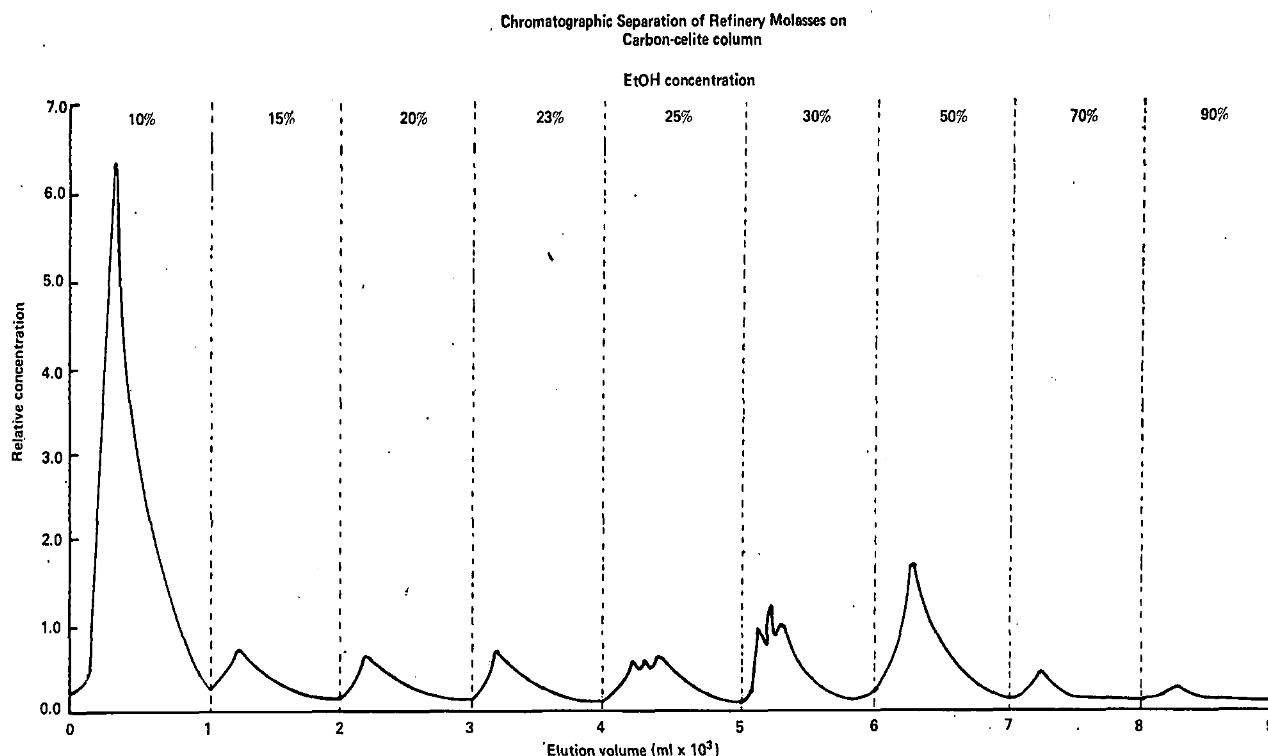


FIGURE 2

TABLE I
$R_{sucrose}$ values of oligosaccharides on Whatman No. 1 paper

Solvent System	A	B	C
Sucrose	1.00	1.00	1.00
Raffinose	0.47	0.46	0.50
1-Kestose	0.69	0.68	0.78
6-Kestose	0.61	0.58	0.69
Peak A	0.72	0.72	0.78
Peak B	0.46	0.48	0.49
Prepared 6-Kestose	1.00*	1.00*	1.00*
	0.67*	0.67*	0.78*
	0.58	0.57	0.67

*Minor spots.

Analysis of peak B gave the following results:—

1. Paper and T.L.C. gave $R_{sucrose}$ values characteristic of raffinose (Table I).
2. Acid hydrolysis yielded glucose, fructose and galactose.
3. Invertase hydrolysis yielded melibiose and fructose.

On the basis of these observations, peak B has been tenatively indentified as raffinose.

Semi-quantitative analysis has shown that peaks A and B are present in refinery molasses in the order of 0.5% by weight.

Acknowledgements

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Discussion

Mr. Alexander (in the chair): We do not know where this investigation will lead us, but it may explain some of the anomalies we have in the sugar industry, such as undetermined losses.

Dr. Matic: A prepared 6-kestose appears to be impure, as shown by the melting point and chromatographic behaviour. This should be borne in mind when further work is carried out.

Mr. Schaffler: The melting point is not as it should be because we only re-crystallised 6-kestose once, owing to lack of time before writing this paper.

Mr. Comrie: In Table I it is shown that 6-kestose contained minor spots and was not as pure as we would have liked. The main spot had an R sucrose value of .58.

Mr. Carter: Can this now be regarded as a routine analysis to be carried out by ordinary laboratory personnel?

Mr. Schaffler: The results reported in this paper are part of a research project which is being carried out to identify oligosaccharides in sugar products.

Once the oligosaccharides have been identified, a routine quantitative thin layer chromatographic method will be adopted.

Initial experiments in this aspect of the work are now in progress.

Mr. Jennings: At the recent ICUMSA meeting, kestoses and disaccharides were discussed and methods mentioned at the previous Copenhagen meeting were tentatively approved.

One difficulty in setting up routine methods for kestoses is to obtain standard samples. We have obtained samples of 1-kestose and 6-kestose from the New York trade laboratory.

Mr. Dutton, of British Sugar Corporation, has done determinations of kestoses and raffinoses in cane molasses from all over the world. The largest amount of kestose and raffinose found in combination was .5% in cane refinery molasses.