

THE ROLE OF OLIGOSACCHARIDES IN CRYSTAL ELONGATION

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Abstract

The class of sugars known as oligosaccharides has been shown to be the principal cause of crystal elongation in the South African industry. Five major oligosaccharide components have been identified and one of these (theandrose) has been synthesised. As far as is known, two trisaccharides - theandrose and erlose - have not been previously reported in cane products. It has been demonstrated that theandrose promotes c-axis elongation. This sugar can be detected in mixed juice and can thus be considered a constituent of the incoming cane.

Introduction

'Crystal elongation' is a term which can be used to describe the shape of a crystal when the ratio of the crystal lengths in any two specified axial directions is larger than that usually observed under similar crystallisation conditions.

Industrial crystallisation takes place under non-equilibrium conditions so that the observed shape of crystals usually depends on the growth rate of the individual faces and, in general, changes occur because growth in certain directions is retarded rather than accelerated. All South African factories and refineries produce elongated sucrose crystals to a greater or lesser extent. The main interest in identifying the causes stems from the associated reduced crystallisation rate. In addition, severely elongated crystals tend to be more fragile and lead to breakage and physical loss.

The relative growth rates of the various faces can be influenced by physical conditions such as temperature, supersaturation and circulation, but chemical factors such as the quality of the non-sucrose components exert a greater influence (Anon, 1985).

Cane sugar crystals are generally elongated in the direction of the c-axis, i.e. growth along the b-axis has been retarded and the crystals assume a fence-post appearance. At times this elongation can be extensive and is often accompanied by processing difficulties. It is widely accepted that dextrans are responsible for the extreme elongation (Atkins and McCowage, 1984). However, there is also some published evidence implicating oligosaccharides in crystal elongation (Kamoda *et al.*, 1968, Montenegro *et al.*, 1983, Smythe, 1967). In addition to these two major groups of compounds, substances such as inorganic salts (Mantovani *et al.*, 1974) and monosaccharides (Vaccari *et al.*, 1989a, Mantovani *et al.*, 1991) have also been shown to influence sucrose crystal morphology.

The SMRI was involved in several investigations during the early 1970s. Although at that stage the evaluation of elongation was very subjective and was not reproducible, early indications were that oligosaccharides rather than polysaccharides were involved (Anon, 1971). Initial emphasis was directed at developing gas chromatographic (gc) procedures to analyse for kestoses (Nurok, 1974, Nurok and Reardon, 1975, Nurok, 1976). A collaborative study with the University of Ferrara (Anon, 1975, 1977) reached the conclusion that of the three kestoses only neo-kestose showed

a slight tendency to c-axis elongation, while the effect was not evident for the other kestoses (Vaccari *et al.*, 1981). Experiments were carried out at 25°C with a non-sucrose to water ratio of 0,02. However, the c/b ratio of about 0,8 is not necessarily a negligible effect (Morel du Boil, 1991). Later work (Anon, 1978) unsuccessfully attempted to correlate length to width (l/w) ratios in massecuite with kestose and dextran concentrations (Bruijn, 1980).

Reproducible laboratory methods for measuring crystal growth rates and crystal shapes, under closely controlled conditions not dissimilar to those prevailing in a factory, were subsequently developed. By selectively removing impurity classes from refinery or factory products, and adding these fractions to pure sucrose solutions, it was found that the oligosaccharide fraction contained the bulk of the elongating and rate-retarding constituents (Morel du Boil, 1986, Bruijn and Morel du Boil, 1986). Under controlled laboratory conditions the extent of elongation was dependent on the concentration of these oligosaccharides. Chemical effects are therefore of greater importance in crystal shape modification than physical parameters (Morel du Boil, 1985).

Once it was established that oligosaccharides as a class were the primary cause of sucrose crystal elongation under local processing conditions, attempts were made to isolate the individual oligosaccharides so their effects on crystal shape could be assessed. The attempts to obtain pure oligosaccharides met with limited success, mainly because of the close similarity of the sugars (Anon, 1985, 1986). Partial separations of the oligosaccharides highlighted the fact that no single constituent was responsible for c-axis elongation and the observed crystal shape resulted from the contributions of several crystal habit modifiers present at varying concentrations (Anon, 1985). Vaccari *et al.*, (1989b) have demonstrated that the crystal shape is modified by successive influences on different crystal faces. This supports the view that several components are involved in crystal elongation.

This paper outlines recent investigations on crystal elongation undertaken at the SMRI and summarises their current standpoint.

Silica gel thin layer chromatography (tlc) has been used to obtain small amounts of the individual oligosaccharides. The main oligosaccharides have been identified using acid hydrolysis and several chromatographic techniques. One of these oligosaccharides has been synthesised and its effect on crystal elongation has been assessed.

Experimental

Most of the experimental procedures have been detailed elsewhere (Morel du Boil, 1985, Bruijn and Morel du Boil, 1986, Morel du Boil and Schaffler, 1990, Morel du Boil, 1991).

Fractionation of impurities

The oligosaccharide components were isolated from factory or refinery products using alcohol classification followed

by carbon column chromatography with step-wise ethanol elution (15% and 50%).

Re-chromatography was carried out on carbon with an ethanol gradient (5 to 20%), or on Fractogel HW 40 (S) with water or on octadecylsilyl with water to obtain partially enriched oligosaccharide fractions.

Isolation of individual oligosaccharides

Small amounts of reasonably pure individual oligosaccharides were obtained by applying about 4 mg of the carbon enriched oligosaccharide fraction to each of four silica gel thin layer plates (Merck 60F₂₅₄ — 5554). The plates were developed for 24 hours in butanol (water saturated): ethanol (100: 40) and, after air drying, the separate oligosaccharides were eluted into 3 ml hypovials using water and were freeze dried.

Micro-scale acid hydrolysis

Individual oligosaccharides (corresponding to 1,6 mg of the original oligosaccharide mixture) were transferred to 3 ml hypovials and 0,015 mg xylose was added as internal standard. The oligosaccharides were hydrolysed in sealed hypovials using 500 μ l 0,1 N trifluoroacetic acid at 60°C for two hours. It had been established that the liberated fructose was not further degraded with these relatively mild hydrolysis conditions, although some oligosaccharides with less labile glycosyl linkages were not hydrolysed beyond the di- and sometimes trisaccharide stage.

Derivatisation and gc of oligosaccharides and hydrolysates

The separate oligosaccharides and the hydrolysis products were freeze dried and trimethylsilyl (TMS) oximes were prepared in the usual way (Schaffler and Morel du Boil, 1984). The TMS derivatives were separated on an HP-5 column (25 m X 0,32 mm X 0,52 μ m), detector 280°C, injector 250°C using one of two temperature programmes:

- (i) 170°C for 2 minutes, programmed at 8°C/min to 270°C for 80 minutes.
- (ii) 170°C for 2 minutes, programmed at 8°C/min to 270°C for 39 minutes, followed by 10°C/min to 300°C for 75 minutes.

Preparation of theanderosse

Theanderosse was prepared using an adaptation of Miki's procedure (Miki *et al.*, 1989). Sucrose (20 g) and dextran T 2000 (2,5 g) were dissolved in a sodium acetate-acetic acid pH 4,3 buffer (50 ml). Enzyme (6 g or 3 000 units of dextranase Novo 50 S) was added and the mixture was maintained at 20°C for 17 hours. The reaction was stopped by boiling the mixture for two minutes.

Sample (50 ml) was applied to the carbon column and eluted using an ethanol gradient. Anion exchange/pulsed amperometric detection (PAD) was used to monitor the separation (Morel du Boil and Schaffler, 1990). The theanderosse enriched fractions were concentrated and re-chromatographed on Fractogel.

Crystal growing and measuring

Previously published methods (Morel du Boil, 1985, Bruijn and Morel du Boil, 1986, Morel du Boil, 1991) were used.

Chromatographic properties of oligosaccharides

Several chromatographic techniques were used to confirm the identity of the main oligosaccharides:

1. Continuous tlc on silica gel using butan-1-ol (water saturated): ethanol (100:40) in an overnight run.

2. Multiple ascent (3 or 4) tlc on silica gel using chloroform: acetic acid: water (3,0:3,5:0,5). Aniline-diphenylamine was used to detect the sugars.
3. Reverse phase high performance liquid chromatography (hplc) using Spherisorb ODS 2 (4,6 X 250 mm, 5 μ m) with water at 0,8 ml/min and RI detection.
4. Hplc on silica (Waters Radial-Pak, 100 X 8 mm ID, 10 μ m) using acetonitrile-water (70:30) containing 0,01% SM2 amine modifier as eluent at 1,0 ml/min with RI detection.
5. Anion exchange hplc (CarboPac PA1) with pulsed amperometric detection (PAD). Eluent was 10 mM sodium acetate: 100 mM sodium hydroxide at 1,0 ml/min. (PAD settings: E1 = 0,05 V (300 mS), E2 = 0,65 V (240 mS), E3 = -0,95 V (360 mS)).
6. Gas chromatography of TMS oxime derivatives (HP 5880 in the splitless mode) with FID detection:
 - (a) on HP-5 (25 m X 0,32 mm, 0,52 μ m) using hydrogen at 35 cm/sec as carrier. This phase is equivalent to SE-54. Sample (3 μ l) was injected at 120°C, with injector at 290°C and detector at 250°C. The temperature was increased to 300°C at 30°C/minute and the valve was opened two minutes after injection.
 - (b) on SGE BP10 (25 m X 0,22 mm, 0,1 μ m) using hydrogen at 40 cm/sec as carrier. This phase is equivalent to OV 1701. Sample (3 μ l) was injected at 120°C, with injector at 250°C and detector at 260°C. The temperature was increased to 250°C at 30°C/minute and the valve was opened two minutes after injection.

Results and discussion

The length to width ratio within the industry

Most factories show an increase in the crystal length to width (l/w) ratio towards the end of the season. The typical trend in this ratio is shown in Figure 1a. However, the ratio can sometimes be high early in the season as well (Figure 1b). Some factories with particularly low ratios frequently show no such seasonal trends (Figure 1c). In the case of raw sugar products the l/w ratio is approximately the same as the c/b ratio. In both factories and refineries the c/b ratio tends to increase as the number of strikes (and hence impurity concentration) increases.

Effect of impurities on crystal elongation

Morel du Boil (1985) showed that most of the crystal elongating properties in a refinery were confined to the oligosaccharide fraction (Table 1). Invertase hydrolysis of this fraction confirmed that the sugar constituents were responsible.

Table 1
Comparison of crystal shape in various refinery molasses fractions (NS/W = 0,1)

Fraction	c/b Ratio
Sucrose	0,54
Molasses	1,33
A - polysaccharides	0,51
B - mono, di & oligosaccharides	1,25
B1 - mono & disaccharides	0,59
B2 - oligosaccharides	1,11

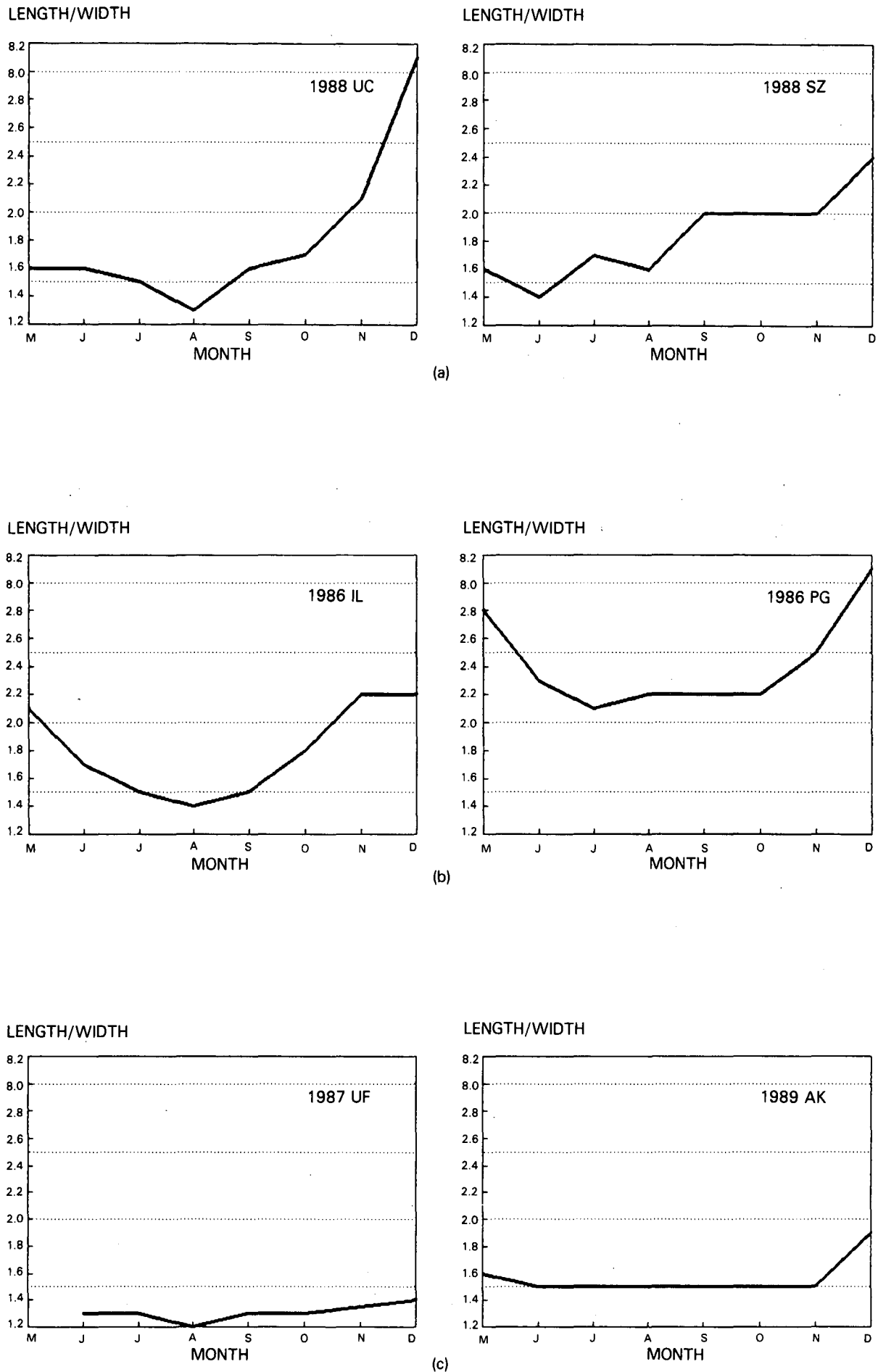


FIGURE 1 Crystal l/w ratio for C-massecurite.

Subsequently run-off molasses samples from five different refineries and molasses samples from three raw sugar factories experiencing crystal elongation (l/w ratios of about 2,5) were divided into two fractions (A - high and B - low relative molecular mass components) using ethanol classification (Bruijn and Morel du Boil, 1986).

Samples from all five refineries showed significant and similar crystal elongation when impurities were present at a non-sucrose to water ratio of 0,1 (Table 2). In all cases the polysaccharides (fraction A) comprised about 7 to 10% of the non-sucrose and played little part in this elongation. The ethanol-soluble components of low relative molecular mass (fraction B) were responsible for practically all the elongation. The oligosaccharides comprised about 10% of the non-sucrose in this fraction.

Table 2

Crystal shape (c/b ratio) in fractionated refinery molasses (NS/W = 0,1, Purity = 97)

Fraction	c/b ratio				
	Refinery				
	1	2	3	4	5
Molasses	2,50	1,54	1,67	1,33	1,33
A - polysaccharides	0,65	0,63	0,67	0,63	0,51
B - mono, di & oligo-saccharides	1,67	1,18	1,25	1,25	1,25
A + B	1,82	1,33	1,67	1,33	1,33

In factory samples much lower purities were necessary in order to get comparable elongations. Although the oligosaccharide concentrations were similar for refinery and factory products, the elongation induced by refinery oligosaccharides was greater than by factory oligosaccharides (c/b = 1,32 compared with c/b = 0,83). This could be due to differences in the type of oligosaccharides present. The polysaccharide concentration for factory products was five times that for refinery products, but the elongation attributable to polysaccharides was similar for both types of product (c/b = 0,62 and c/b = 0,70) (Table 3).

Thus oligosaccharides are also the main cause of factory derived elongation although the elongation attributable to polysaccharides can be as significant.

Table 3

Crystal shape (c/b ratio) in fractionated factory molasses (NS/W = 0,5, Purity = 86)

Fraction	c/b ratio		
	Factory		
	1	2	3
Molasses	0,91	1,00	1,25
A - polysaccharides	0,77	0,69	0,63
B - mono, di & oligo-saccharides	0,83	0,83	0,83

Enriched oligosaccharide components and their effect on crystal shape

The oligosaccharide fraction (B2 - see Table 1) was sub-divided into B2.1 and B2.2 using 15% and 50% ethanol. Fraction B2.1 showed more elongating potential (c/b = 0,77) than B2.2 (c/b = 0,61) and was further sub-divided, with fraction B2.1.1 (10% ethanol) showing more elongation (c/b = 0,69) than B2.1.2 (15% ethanol and c/b = 0,57). However, none of these fractions was pure and all showed some elongating potential. Clearly no single constituent was responsible for the elongation and several crystal habit modifiers contributed to the observed crystal shape. Vaccari *et al.*, (1989b) have recently shown that the crystal shape in cane processing is modified by successive influences on different crystal faces. This observation supports the view that several components are involved in crystal elongation.

Tlc isolation, acid hydrolysis and chromatography of oligosaccharides

The gc chromatograms of the partially enriched oligosaccharide sub-fractions (B2.1.1 and B2.1.2), before and after mild acid hydrolysis, are shown in Figures 2 and 3. With these conditions all the trisaccharides in B2.1.1 are at least partially hydrolysed, but an acid resistant disaccharide remains. In the case of B2.1.2 at least one oligosaccharide was not hydrolysed and it is clear from the complex disaccharide spectrum that many of the oligosaccharides are only partially hydrolysed with these mild conditions.

Peaks in the oligosaccharide mixture have been arbitrarily numbered according to the elution order on silica gel. The sugar components (tentatively identified by gc retention) of each are summarised in Table 4.

Table 4

Component sugars of oligosaccharides

Peak	Tlc R _S *	Gc R _{Xyl} *	Component sugars		Ratio
			Monosaccharides	Disaccharides	
6	0,76	7,03	F and G		2:1
7	0,71	7,23	F and G		2:1
8a	0,61	7,63	F	Maltose	1:1
8b	0,61	7,84	G	Cellobiose	1:1
9	0,50	7,21	F and G		2:1
10	0,44	8,90	F	iso-Maltose	1:1
12	0,36	7,02	F	Melibiose	1:1

* R_S = retention time relative to that of sucrose
R_{Xyl} = retention time relative to that of Xylose

In Table 5 the chromatographic behaviour of several reference oligosaccharides is compared with that of some of the oligosaccharides isolated from refinery molasses.

Results presented in Tables 4 and 5 confirm the identity of the five major oligosaccharides as:

- Peak 9 1-kestose
- Peak 10 theanderose
- Peak 7 neo-kestose
- Peak 8a erlose
- Peak 12 raffinose

As far as is known this is the first time that theanderose (Morel du Boil, 1991) and erlose have been reported in cane products. Both are known as constituents of honey (Siddiqui, 1970, Low and Sporns, 1988) and have been described as products of enzymic syntheses (White and Maher, 1953, Barker *et al.*, 1957).

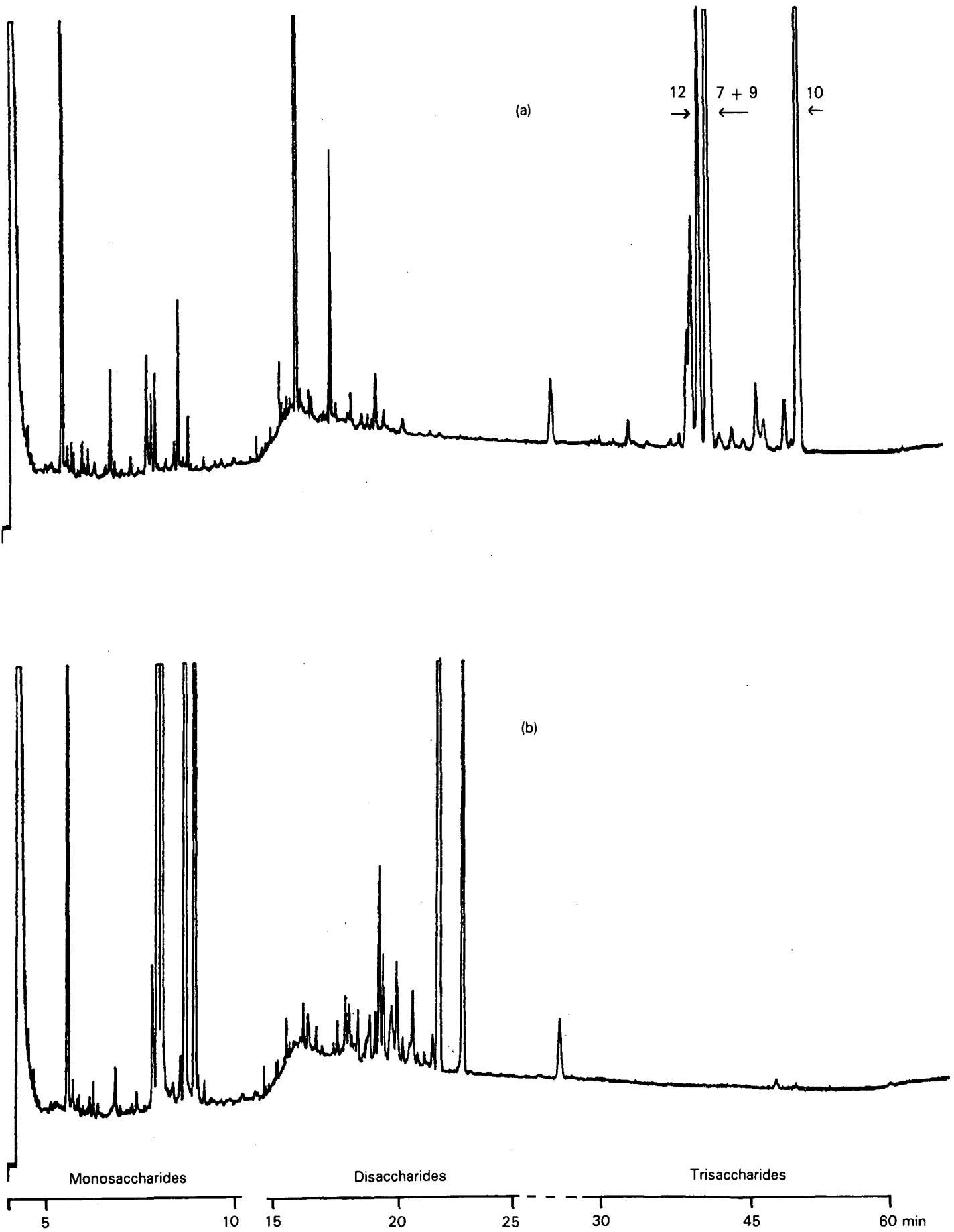


FIGURE 2 Gas chromatograms of
(a) fraction B2.1.1
(b) fraction B2.1.1 after mild acid hydrolysis.

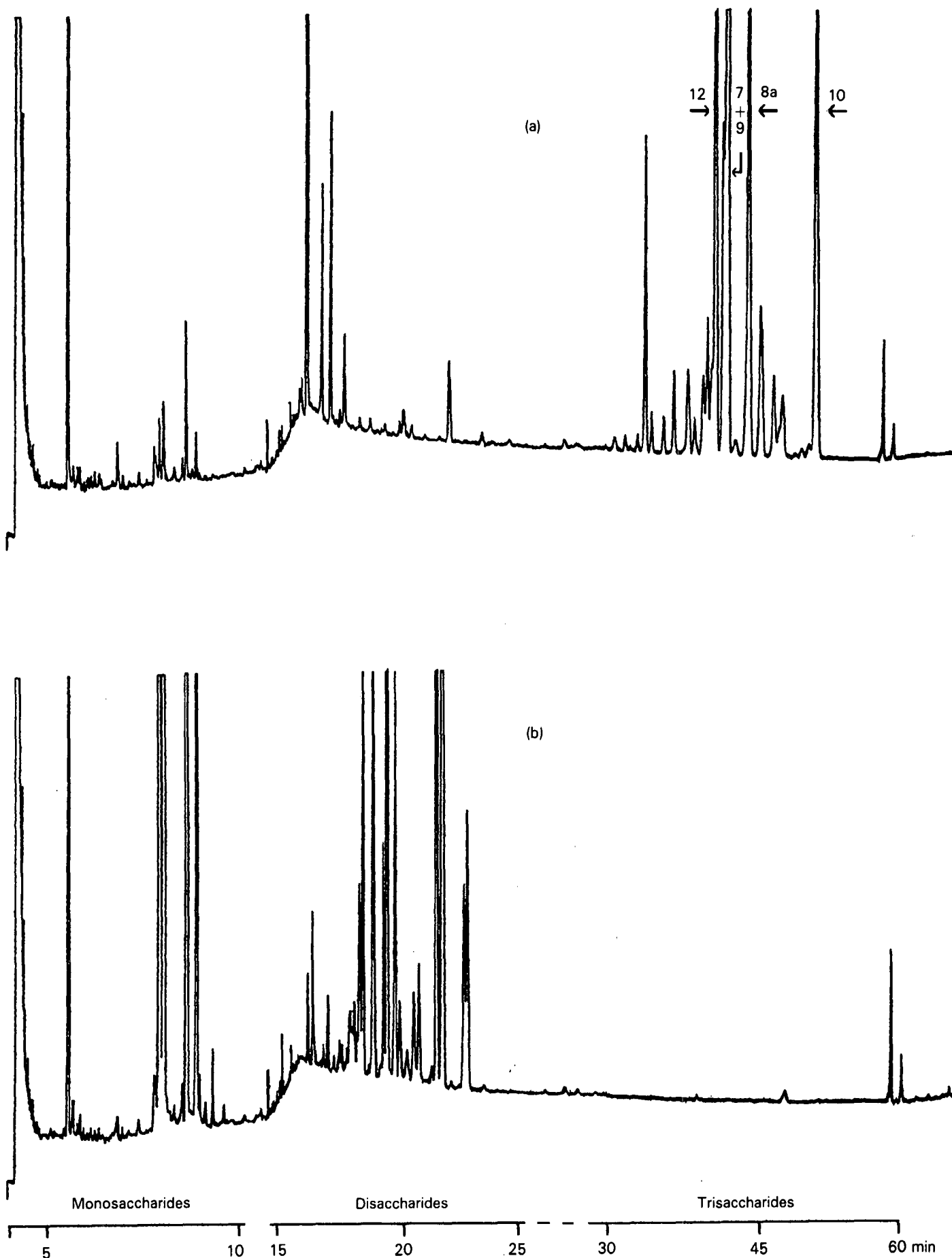


FIGURE 3 Gas chromatograms of
(a) fraction B2.1.2
(b) fraction B2.1.2 after mild acid hydrolysis.

Table 5
Comparison of chromatographic properties of molasses isolates

Method	Tlc	Tlc	Hplc	Hplc	Hplc	Gc	Gc
Conditions *	1	2	3	4	5	6a	6b
Retention	R _s	R _s	R _T (min)	R _T (min)	R _T (min)	R _T (min)	R _T (min)
Carbohydrate:							
Peak # 6	0,72	0,62	(5,96)	13,11	-	11,81	23,01
Peak # 7	0,69	0,61	8,05	13,02	26,02	12,09	23,78
Peak # 8	0,59	0,59	5,42	13,92	14,85	12,45	25,64
			(5,92)		16,96		26,84
Peak # 9	0,48	0,50	5,93	14,05	11,32	12,08	23,62
Peak # 10	0,42	0,55	5,73	14,75	12,09	13,56	31,54
Peak # 12	0,33	0,47	5,61	15,97	9,83	11,90	22,94
Theanderose	0,42	0,55	5,71	14,63	12,06	13,55	31,54
Erlöse	0,58	na ⁺	na	na	na	12,46	na
1-Kestose	0,47	0,52	5,96	14,09	11,41	12,08	23,42
6-Kestose	0,43	0,47	3,89	15,20	20,84	12,04	23,31
neo-Kestose	0,68	0,63	8,03	13,01	25,79	12,13	24,02
Raffinose	0,34	0,50	5,59	15,89	9,80	11,92	23,06

* Chromatographic conditions described in text

+ na = not available

Raffinose is not normally reported in cane products and a popularly held belief is that raffinose is absent. However, Gross *et al.*, (1962) confirmed the presence of raffinose in cane products and estimated the level at about 0,017% in a Natal raw sugar. Binkley (1964) isolated and identified raffinose from cane molasses. Schiweck and Busching (1970) and Gross (1970) found about 0,03% in a South African raw sugar, about 0,012% in a South African refined sugar and about 0,1% in South African refinery molasses. Morel du Boil *et al.*, (1970) have previously demonstrated the presence of raffinose in South African refinery molasses. According to Gross *et al.*, (1962) raffinose contents in cane sugars are about one-tenth or less of those shown by the corresponding beet sugars. Schiweck and Busching (1970) claim that raffinose determinations do not enable a white beet sugar to be distinguished from a white cane sugar since the raffinose content of a good quality beet sugar does not differ markedly from that of a white cane sugar. The influence of raffinose on b-axis elongation is well documented (Vaccari *et al.*, 1986).

Since Nurok (Anon., 1977) and Vaccari *et al.*, (1981) had previously concluded that the kestoses made minimal contribution to c-axis elongation, it was decided to synthesise theanderose and to examine its role in crystal elongation.

Preparation of theanderose

Only some of the commercially available dextranases synthesised theanderose. Reaction conditions were adjusted to give the best ratio of theanderose to kestoses. Despite the fact that all theanderose preparations were associated with large amounts of sucrose and all three kestoses in addition to iso-maltose and iso-maltotriose and some minor compounds, the oligosaccharide spectrum was considerably simpler than those observed with cane factory products. Theanderose of about 90% purity was obtained after chromatography on carbon followed by re-chromatography on Fractogel.

Effect of theanderose on crystal shape

Crystals were grown in pure sucrose solutions and in solutions spiked with refinery molasses, refinery molasses oligosaccharides or theanderose. The mean c/b ratios for the

four sets of crystals are presented in Table 6. The theanderose concentration is comparable with, but probably higher than, that encountered in exhaust molasses.

Table 6
Sucrose crystal shape in the presence of theanderose

Growth medium	NS/W	Olig/W	Th/W	c/b
Sucrose				0,60
Sucrose + theanderose	0,005	0,005	0,005	0,85
Sucrose + molasses	0,013	0,013	0,002	1,25
oligosaccharides				
Sucrose + molasses	0,101	0,013	0,002	1,30

Theanderose contributes to c-axis elongation, but is clearly not the only factor involved. Obviously other oligosaccharides (and perhaps other effects) are also implicated. Theanderose at this level accounts for about one third of the elongation observed with molasses oligosaccharides.

Theanderose has been found at about 0,5% in refinery molasses, about 0,05 to 0,08% in C-molasses and two random mixed juice samples contained about 50 ppm.

It is obvious that the extent of elongation observed in the factory is the result of several contributing factors. The relative importance of different oligosaccharides depends on their potency as crystal growth inhibitors and on their relative concentrations. Crystal growth conditions such as temperature and saturation probably also exert an influence.

The role played by other oligosaccharides needs to be reassessed under comparable experimental conditions and at levels realistically related to those of cane products. For example, although Nurok (Anon, 1977) and Vaccari *et al.*, (1981) found the effect of kestoses negligible, an elongation ratio of 0,8 cannot be disregarded. In addition, their crystallisation conditions were far removed from those encountered in the factory.

With the implementation of improved analytical techniques such as anion exchange hplc coupled with pulsed amperometric detection and of capillary gc in the splitless

mode, it is now more feasible to monitor oligosaccharide contents in various factory products. Such monitoring should enable the importance of seasonal effects and of factory to factory differences to be established.

Conclusions

It has been shown that most of the crystal elongation encountered in cane processing can be attributed to the presence of oligosaccharides and that no single component is totally responsible. The main oligosaccharides in cane molasses have been identified as 1-kestose, theanderose, neokestose, erlose and raffinose. Theanderose and erlose have not previously been reported in cane products. Sufficient theanderose (6- α -glucopyranosyl-sucrose) has been synthesised to allow its effect on c-axis elongation to be demonstrated. It has been shown that theanderose contributes to c-axis elongation, particularly in the refinery. The effect of the other oligosaccharides on c-axis elongation should be re-assessed using the same reproducible technique. It is possible that polysaccharides have some effect on crystal elongation in the raw sugar factory. During the coming season it is planned to monitor the concentrations of the different oligosaccharides and of dextran in various factory streams to establish seasonal and inter-factory variations.

Acknowledgements

The authentic theanderose and erlose were gifts from Prof. Olof Theander of the Swedish University of Agricultural Sciences, Uppsala, Sweden, and Dr S Chiba of the Faculty of Agriculture, Hokkaido University, Sapporo, Japan, respectively. The assistance of Eshara Ramphal, Danielle de Gaye and Jo Day-Lewis in preparing and running many of the gc samples is appreciated. Technicians in the Processing Division at the SMRI carried out the crystal shape measurements for the industrial survey.

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