

AN ENZYMIC-HPAEC PROTOCOL FOR THE ANALYSIS OF POLYSACCHARIDES IN SUGARCANE PRODUCTS - DEXTRAN AND SARKARAN

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Abstract

An analytical scheme for the partial differentiation of sugarcane polysaccharides is presented. The procedure is based on the isolation of high molecular weight material, followed by selective hydrolysis of the polysaccharide with industrial hydrolases. Characteristic oligosaccharide products are analysed using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The protocol has been used to monitor seasonal and geographic differences in the polysaccharide distribution for juices and sugars. Comparisons with some empirical procedures are also made.

Keywords: polysaccharides, dextrans, sarkaran, enzyme, HPAEC-PAD

Introduction

Dextran is a polysaccharide present in cane products as the direct result of cane deterioration. Its presence has an adverse effect on cane processing due to viscosity increases and also indicates that sucrose has been destroyed. The effects will be seen from extraction through to exhaustion and crystallisation (Atkins and McCowage, 1984). Although the presence of dextran and some of its processing effects have been recognised since the early days of sugar processing, its importance was highlighted by the Australian industry when mechanical harvesting of chopped cane was introduced. Over the years various analytical procedures have been proposed (Geronimos and Greenfield, 1978; Goodacre and Martin, 1981; Curtin and McCowage, 1986; Curtin, 1988; Sarkar *et al.*, 1991; Day and Plhak, 1998). The only two to have gained widespread acceptance and applicability have been the original "haze" procedure introduced by Nicholson and Horsley (1959) and the AOAC (or Roberts) copper sulphate method (Roberts, 1983). The former has undergone many modifications in an attempt to improve its reliability and specificity (McCowage, 1994). However, whilst well aware of its shortcomings, it served the Australian industry well in its drive to improve processability of mechanically harvested cane (Atkins and McCowage, 1984) and is, to date, probably the simplest routine procedure for predicting processing or refining characteristics (Urquhart *et al.*, 1993).

Sarkaran is a polysaccharide which has been reported in both stale (as opposed to deteriorated) cane and standover cane (*i.e.* cane harvested in the second season of growth). It is not clear whether it is present as a result of normal cane physiology involving natural enzymes, or as a result of fermentation associated with contaminant yeasts or fungi. It belongs to a family of polysaccharides known as pullulans. Pullulans are extracellular α -glucans elaborated by yeast-like fungi such as *Aureobasidium pullulans*. Structural investigations have indicated that there is

no unique structure for this material. The name pullulan is to be regarded as a generic name for a group of similar polysaccharides. Pullulans are essentially linear molecules in which α -maltotriose residues are polymerised endwise through α -1:6 links. However they also contain variable amounts of maltotetraose linked through the ends by 1:6 bonds to maltotriose residues (Catley *et al.*, 1966). Such compounds have been reported in sugarcane and molasses (Nicholson and Lilienthal, 1959; Sutherland, 1960; Bruijn, 1966a, 1966b, 1970; Blake and Littlemore, 1984a, 1984b; Blake and Clarke, 1984a, 1984b). Bruijn named this cane polysaccharide sarkaran. Estimates of molecular size ranged from 50 000 to 200 000 Da (Bruijn, 1966b; Blake and Clarke, 1984b). Typical problems associated with processing standover cane include very high syrup viscosities, poor crystallisation rates and gumming of heating surfaces. Both dextran and sarkaran form part of the gums.

Enzymes offer an analytical route for differentiating between polysaccharides. Although enzymes can be extremely specific, their use is limited by the availability of suitably pure enzymes and by their usually restricted range of reaction conditions (e.g. pH, temperature, substrate concentration). Brown and Inkerman (1992) published a procedure for measuring dextran using a commercial dextranase and HPLC. Because of the relative insensitive refractive index (RI) detection used, the method was rather cumbersome and included ion exchange and concentration steps.

Anion exchange chromatography coupled with pulsed amperometric detection has gained popularity as a powerful, selective and sensitive analytical technique for mono- and oligosaccharides. By combining dextranase hydrolysis with the sensitivity and specificity of HPAEC-PAD chromatography much of the time-consuming work-up procedures have become unnecessary (Anon., 1999; Zimmer *et al.*, 1999).

The technique is no more rapid than the Roberts procedure for example, but does afford a greater degree of specificity. The protocol has been expanded to include the measurement of sarkaran.

Blake and Clarke (1984a) described a colorimetric assay using a commercially available pullulanase. The procedure was prone to error because of the presence of background sugars.

Experimental

Analytical methods:

- *Dextran and sarkaran (enzymic)* Polysaccharide material was isolated using ethanol precipitation. The crude mixture was hydrolysed with either dextranase or pullulanase and the characteristic oligosaccharide products (isomaltotriose

in the case of dextran or maltotriose and maltotetraose in the case of sarkaran) were measured using high performance anion exchange chromatography (HPAEC). Details are given in Appendix 1.

- *Dextran (Roberts)* Sugars were analysed according to the Official AOAC method (Clarke and Godshall, 1988). Roberts (1983) procedure was used for juices.
- *Dextran (haze)* The Official ICUMSA procedure was used for sugars (Anon., 1994). Juices were analysed using Method 28 of the Australian laboratory manual (Anon., 1991).
- *Starch* This was analysed in accordance with the SASTA manual (Anon., 1985).
- *Gums* The method in the SASTA manual was followed (Anon., 1985).

Samples The following sample sets were investigated:

- *Mixed juice*
 - (a) The weekly mill composite mixed juice samples supplied frozen by Cane Testing Services (CTS) during the 1999/00 season. Samples for weeks 24 (14/8/99) to 39 (27/11/99) from selected mills were used in this survey.
 - (b) These samples were subsequently combined to give six characteristic composites for further investigation. Composites 1a, 2a and 3a covered the period 4/9 to 2/10 for KM and ML, NB and UC, SZ respectively, whilst composites 1b, 2b and 3b covered the period 9/10 to 13/11 for the same mill combinations.
- *Raw sugar*
 - (a) VHP from the 1997 season and export raws from the 1998 season.
 - (b) VHP sugar received by the South African Sugar Terminals (SAST) during the 1999/00 season corresponding to the period for which mixed juice was analysed.

- *Refined sugar*

Weekly refined sugar composites from NB for selected periods during the 1999/00 season.

- *Direct analysis of cane (DAC) extracts*

Several DAC extracts obtained during a cane deterioration trial carried out at PG during January and February 2000 were analysed (Barker, 2000).

- *Final molasses*

Molasses samples from UC during October 1999 were analysed.

Results and discussion

Method evaluation

Isolation of polysaccharides: An important prerequisite for the measurement of almost any impurity in sugar products is the separation of the impurity from the large sucrose matrix. In the case of polysaccharides their insolubility in alcohols has been the basis of most traditional procedures. In addition, this isolation procedure is flexible - by varying the alcohol concen-

tration (e.g. 50 or 80%) it is possible to fractionate different molecular weight (M Wt) ranges. Ethanol precipitation was used in this study. This step proved to be tedious and created a severe bottleneck. Other techniques (e.g. size exclusion or ultrafiltration) are available and are being used increasingly (Zimmer *et al.*, 1999). However, there are limitations and drawbacks to these methods. A distinct disadvantage in the case of size exclusion on an analytical scale is that it is only possible to remove the bulk of the sucrose without any molecular weight distinction. It has become customary to use acidified ethanol to isolate 'gums' (Boyes and Wilson, 1964; Jennings, 1964). These acidified alcohol insoluble materials include various polysaccharides, e.g. dextrans, starch and hemicelluloses, as well as inorganic ash, organic acids (depending on the pH), proteinaceous substances and sometimes wax. The proteins can be precipitated with trichloroacetic acid (TCA) and filtered off before the alcohol precipitation. The AOAC procedure for raw cane sugar makes no mention of TCA addition (Clarke and Godshall, 1988). Although Roberts (1983) claims that the procedure is applicable to other products, no filtration is carried out after adding TCA and so the addition of TCA serves merely to decrease the pH.

Because of the specificity of the enzyme assays used in this investigation, the non-removal of protein is irrelevant. However, results presented in Tables 1 to 3 indicate quite clearly that the recovery of standard dextran is more complete at lower pH. Furthermore, both dextran and sarkaran results are higher and more precise at the lower pH for standards and juice. With sugar there is no apparent difference. It is obviously important that the isolation procedure is carried out at low pH.

Enzyme selection: Industrial, rather than analytical, enzymes were selected on several grounds:

- the extremely high purity of analytical enzymes was not deemed necessary - the objective was not to carry out detailed structural studies, but rather to establish the 'dextran' nature of the polysaccharides
- suitable industrial enzymes were available
 - dextranase from *Chaetomium gracile* (Genencor Dextranex L-4000¹) was used (Anon., 1996). Brown and Inkerman (1992) confirmed that this fungal enzyme hydrolysed cane dextrans faster and to a greater extent than other dextranases such as the analytical dextranase derived from *Penicillium sp.* Furthermore, the ma-

Table 1. Effect of TCA on polysaccharide recovery for dextran (T-500) or pullulan (Sigma) using ethanolic isolation and enzymic-HPAEC.

	Dextran recovered (%)		Pullulan recovered (%)	
	with TCA	without TCA	with TCA	without TCA
Range	95 - 97	50 - 85	100 - 105	50 - 90
Mean	95.8	71.9	102.8	76.7
rsd (%)	1.5	27	2.6	27

Table 2. Effect of TCA on polysaccharide recovery for mixed juice (n = 6) using ethanolic isolation.

Sample	Dextran (mg/kg Bx)			Sarkaran (mg/kg Bx)		
	with TCA	without TCA	Relative recovery (%)	with TCA	without TCA	Relative recovery (%)
1	110	70	64	230	170	74
2	145	100	69	160	110	69
3	265	225	85	1 825	1 455	80
4	540	465	86	2 045	1 600	78
5	720	600	83	585	310	53
6	1 465	1 145	78	550	365	63
Mean	540	435	78	900	670	70
sd			9			10

for hydrolysis product is isomaltose, with minor amounts of isomaltotriose and some glucose. In contrast, bacterial dextranases (e.g. *Bacillus sp*) give a more complex oligosaccharide spectrum, making quantitation more difficult. Fewer reaction products give better sensitivity.

- pullulanase from *Bacillus acidopullulyticus* (Novo Promozyme 200L) was used (Anon., 1989). The only hydrolysis product from standard pullulan was maltotriose. The precipitated cane polysaccharide (sarkaran) yielded maltotriose and maltotetraose on complete hydrolysis.

- both enzymes were shown to be free of interfering activities for this application.

A further advantage in the use of industrial enzymes in an analytical role is their ready transferability to pilot or factory trials if required. Although it is usually difficult to select suitable factory conditions for optimum enzyme activity and the economic feasibility is frequently questionable, the analytical results can be used as a direct indication for the potential for successful removal in sugar processing.

Analytical conditions: HPAEC and hydrolysis conditions are discussed.

● *Optimisation of HPAEC conditions - Dextran*

Isocratic conditions were established such that maximum separation between isomaltose and residual sucrose and between residual sucrose and cellobiose (initially selected as a suitable internal standard) was achieved. It was also important to ensure that oligosaccharides such as the malto-homologues or unreacted isomaltotriose were resolved. Furthermore the enzyme carrier, glycerol, eluted as an overloaded peak close to the column void volume. Given these constraints, 250 mM sodium hydroxide was the best compromise.

● *Optimisation of HPAEC conditions - Sarkaran*

The enzymic hydrolysate from sarkaran will contain approximately equimolar amounts of maltotriose and maltotetraose as well as limit dextrans from the partial degradation of amylopectin. Such a molecular size spectrum necessitates the use of a solvent gradient. The solvent programme selected maintained the selectivity established for the dextran hydrolysate, but ensured that higher oligosaccharides did not interfere with subsequent samples.

Table 3. Effect of TCA on polysaccharide recovery for raw sugar (n = 3) using ethanolic isolation.

Sample	Dextran (mg/kg)			Sarkaran (mg/kg)		
	with TCA	without TCA	Relative recovery (%)	with TCA	without TCA	Relative recovery (%)
1	110	110	100	30	40	133
2	90	95	106	160	165	103
3	355	375	106	40	50	125
Mean			104			120
sd			3			16

● *Optimisation of hydrolysis conditions - Dextran*

The hydrolysis was carried out in unbuffered solution to avoid the later removal of large salt concentrations. In order to maintain simplicity for a semi-routine procedure the enzyme was used as supplied in the glycerol medium. As the procedure was being developed for confirmatory purposes it was also desirable that the hydrolysis be driven to completion *i.e.* maximum isomaltose and minimum isomaltotriose in the hydrolysate. The formation of isomaltose and isomaltotriose was rapid. The subsequent breakdown of isomaltotriose was slow. A compromise between excessive tailing from the overloaded glycerol peak and the time required for the disappearance of the isomaltotriose peak, resulted in the decision to use overnight hydrolysis. This was not seen as a disadvantage, since the lengthy isolation procedure prevented the total analysis being completed in a single day. The enzyme as supplied had lost some activity (nominally initially 4 000 DXU/g, where one dextranase unit (DXU) yields the equivalent of 0.009 μ moles of glucose per minute), and an enzyme dosage of about 480 DXU (nominal) was used for 20 ml aliquots of dextran solution (up to 2 mg). There was no difference in the reaction rate at 44°C or 55°C. The enzyme exhibited enhanced amylase activity at 55°C and so 44°C was used for hydrolysis. The enzyme was inactivated by boiling for two minutes. The isomaltose calibration was generated by parallel hydrolysis of standard dextran and the absence of isomaltotriose was used as confirmation that hydrolysis was complete and that the enzyme dosage was adequate.

Table 4. Calibration parameters for dextran and sarkaran.

	Dextran (Peak Height)	Pullulan (Peak area)
slope	29 313	397 057
SE	126	1283
r ²	0.9999	0.9999
df	6	6
Range (mg/l)	1.5 to 80	1 to 20

● *Hydrolysis conditions - Pullulan*

The incubation conditions were not optimised. The supplier's data sheet was used as a guideline in establishing conditions. The reaction was carried out at solution pH (slightly acidic) to avoid excessive buffer salt concentrations. The nominal activity of Promozyme L - 200 (Novo) was 200 PUN/g where one pullulanase unit Novo (PUN) liberates the equivalent of 1 μ mole of glucose per minute. The reaction was carried out at 55°C where the enzyme is expected to retain about 90% activity using an enzyme dosage of about 3.75 PUN for 20 ml aliquots of pullulan standard (up to 0.5 mg). The enzyme was inactivated by heating to boiling for two minutes. A reaction time of 60 minutes was considered suitable for complete hydrolysis under these conditions since no further hydrolysis was observed with a 90 minute reaction time.

Linearity: The procedures have been shown to be linear in the range 1.5 to 80 mg/l for dextran and 1 to 20 mg/l for pullulan (Table 4). This corresponds to about 50 to 2 500 mg/kg dextran on raw sugar and about 50 to 1 250 mg/kg sarkaran on raw sugar using the recommended sample dilutions (Appendix 1). Although concentrations of about 5 mg/kg are readily detected, linearity has not been checked in this region.

Reproducibility and repeatability: The precision of the methods was estimated by analysing six raw sugar samples in duplicate on two occasions. The statistics for this intralaboratory comparison are summarised in Table 5. The raw data are included in Appendix 2. Data from previous interlaboratory studies on the haze (McCowage, 1994) and Roberts (Clarke and Godshall, 1988) procedures are also included in Table 5. Both the reproducibility (R) and repeatability (r) compare favourably with these larger studies. The repeatability is also in agreement with that quoted by Galea and Inkerman (1993) for a similar enzymic assay - repeatability relative standard deviation (RSD(r)) < 3.2%. The RSD (r) for sarkaran is considerably higher - this is almost certainly due to the relatively low levels of sarkaran and the limited range of values included here.

Internal standardisation: It is not possible to include an internal standard for the complete analysis. Brown and Inkerman (1992) have discussed the advantages of including an internal

Table 5. Summary statistics for repeatability and reproducibility, using raw sugar samples (n = 6, analysed in duplicate).

	Dextran (enzyme)	Sarkaran (enzyme)	Dextran (haze) ¹	Dextran (AOAC) ²
Range (mg/kg)	45 - 920	0 - 70	10 - 600	370 - 1 000
Mean	360	41	280	574
Samples	6	6	7	4
Days/Labs	2 (days)	2 (days)	9 (labs)	13 (labs)
S(r)	12	6	15	24.6
S(R)	29	7	29	75.8
RSD(r) (%)	3.4	15	5.4	4.3
RSD(R) (%)	8	17	10.4	13.2

1. Data from McCowage, 1994

2. Data from Clarke and Godshall, 1988

S(r) = repeatability

S(R) = reproducibility

standard after precipitation of the polysaccharides. Cellobiose had previously been used as internal standard (Anon., 1999), but was found to hydrolyse slowly. In this application raffinose was considered a more suitable internal standard. It was not hydrolysed by either enzyme and eluted in a convenient section of the chromatogram. Analysis of 190 samples showed that there was no significant difference between internal and external standardisation ($t = 0.88 < t_{crit} = 1.97, \alpha = 0.5, n = 190$). With reasonable analytical expertise, internal standardisation is considered to be an unnecessary complication and its use has been discontinued. Errors associated with incomplete recovery during the initial polysaccharide isolation are a more likely source of bias.

Applications

The purpose of this work was not to develop yet another analytical procedure for estimating 'dextran', but rather to provide a confirmatory tool in cases where the routine AOAC (Roberts) or haze procedures were in question for some or other reason. The extension to include sarkaran arose because of viscosity problems experienced at UC during October, 1999. The specificity of the procedures has been used to differentiate polysaccharide components and some applications are discussed below.

Table 6. Polysaccharides (mg/kg) in export and VHP sugar.

	Gums	Dextran (AOAC)	Dextran (enzymic)		Sarkaran
			Total	High MWt	
VHP - 1997	450	200	350	300	0
	900	280	145	-	40
	750	320	180	85	20
	1 400	360	105	35	35
	1 250	430	105	65	195
	850	450	140	-	40
	2 000	500	455	370	-
	2 000	500	325	310	40
	850	510	380	-	40
	1 050	580	365	235	20
	1 350	640	470	340	-
	1 650	750	290	205	55
	1 550	850	825	645	-
1 550	850	985	810	55	
Export raw - 1998		300	135	-	-
		350	135	70	-
		430	180	75	-
		660	350	260	-
		685	455	310	-
		735	530	-	-
		960	625	510	-
		960	635	505	-

● Export raw and VHP sugars - total vs high molecular weight dextran

One of the major differences between the AOAC method and the haze technique is the molecular weight range of polysaccharides included. Polysaccharides were isolated from 22 samples of sugar using both 50% (high molecular weight fraction) and 80% ethanol (total polysaccharides). Results from a selection of sugars produced in 1997 and 1998 are presented in Table 6. Not unexpectedly, there is a large variation in the proportion of total dextran that is high molecular weight (30 to 95%) and a poor correlation between high MWt dextran and the AOAC procedure ($r^2 = 0.557, n = 17$). Similar trends have been reported frequently and the results simply confirm the inherent inadequacy of the AOAC technique as a useful predictor of refinery performance where high M Wt dextrans are considered more troublesome. There are no obvious relationships between the gums measured and any of the individual polysaccharides. Sarkaran is present in most of the VHP sugars analysed.

● Mixed juice (weekly)

Samples from six mills were analysed for dextran on a weekly basis from September to November. Sarkaran analyses were

Table 7. Polysaccharide analyses on MJ composites.

MJ composite	Gums (mg/kg Bx)	Dextran (AOAC) (mg/kg Bx)	Dextran (D) (enzyme) (mg/kg Bx)	Sarkaran (S) (enzyme) (mg/kg Bx)	Starch (St) (mg/kg Bx)	(D+S+St) (%Gums)
1a	2 000	1 800	110	230	220	28
1b	2 100	2 100	145	160	270	27
2a	5 100	2 700	265	1 825	260	46
2b	5 350	3 500	540	2 045	270	53
3a	5 300	3 900	720	585	940	42
3b	5 800	4 300	1 465	550	860	49

Table 8. Measurement of specific polysaccharide before and after removal of accompanying polysaccharide (Mean result from 6 composites).

	Before removal	After removal of:		
		Starch	Sarkaran	Dextran
Dextran	540	510	545	0
Sarkaran	900	850	0	840
Starch	470	0	565	-

later included for part of this period. The available data are included in Appendix 3. There is an increase in both dextran and sarkaran later in the season, but the mills clearly fall into three groups:

- (1) relatively low dextran and sarkaran throughout (*e.g.* ML, KM, AK)
- (2) moderately high dextran throughout, with very high levels of sarkaran later in the season (*e.g.* NB, UC)

Table 9. Polysaccharides (mg/kg) in VHP sugar.

Mill	Month	Gums	Dextran (AOAC)	Dextran (haze)	Dextran (D) (enzyme)		Sarkaran (S) (enzyme)		D + S (HMWt)	Starch
					Total	HMWt	Total	HMWt		
KM	Sept	500	155	10	70	25	0	0	25	60
	Nov	550	290	5	65	20	10	0	20	80
AK	Sept	700	205	20	140	45	30	0	45	100
	Oct	-	240	15	110	45	25	0	45	105
	Nov	750	375	60	245	85	45	0	85	95
UC	Sept	850	195	70	90	-	155	-	-	135
	Oct	-	270	75	90	40	160	45	85	135
	Nov	1 450	675	285	380	200	300	60	260	150
SZ	Sept	900	415	165	335	125	50	0	125	140
	Oct	-	520	185	355	180	40	5	185	125
	Nov	1 250	810	395	785	345	75	5	350	110

(3) very high dextran later in the season together with moderate, but unchanged levels of sarkaran (*e.g.* SZ).

● *Mixed juice (composites)*

In the light of the above trends the juices were split into two periods for the three groups (see Experimental) and several empirical polysaccharide analyses were carried out. From Table 7 it can be seen that the AOAC procedure includes considerable non-dextran material and approximates the gums analysis. This may be partly related to the method of TCA addition. There is no obvious relationship between the AOAC results and the much lower enzyme results for dextran. The differences between the areas are striking - with high sarkaran levels in group 2 and high dextran and starch in group 3. The specificity of the enzyme assays was demonstrated by showing that enzymic removal of potential interferents had little effect on the analysis (Table 8).

● *VHP sugar*

VHP sugar delivered to the Terminal from these three mill groups during this period was analysed. Results are given in Table 9. Sarkaran is the predominant polysaccharide in the VHP pro-

Table 10. Distribution of sarkaran input between sugar and molasses (October, 1999).

Sarkaran in:	Mixed juice (tons)	16.1
	Sugar (tons)	1.4
	Molasses (tons)	15.7

Table 11. Polysaccharides (mg/kg) in refined sugar - NB 1999.

WE	Dextran (haze)	Dextran (enzyme)	Sarkaran (enzyme)
17/4	140	150	0
24/4	85	140	0
1/5	110	125	0
15/5	20	85	0
25/7	5	30	0
29/8	10	35	10
19/9	20	50	15
31/10	45	75	15
7/11	105	145	20
21/11	145	175	20
28/11	100	135	10
5/12	40	70	0

duced in the Midlands and some of this is high MWt. Almost half the dextran in VHP from SZ is high M Wt. The agreement between the haze dextran and the enzymic dextran for high M Wt dextrans was good ($r^2 = 0.95$, $n = 11$, with slope = 0.77).

An estimate of the distribution of sarkaran between final molasses and VHP produced during October, 1999 shows that about 9% of the sarkaran input is found in the sugar crystal (Table 10). This is comparable to published figures of 10% transfer for dextran (Day, 1984).

● *Refined sugar*

The data for NB refined sugar are presented in Table 11. There was excellent correlation between the haze procedure and the

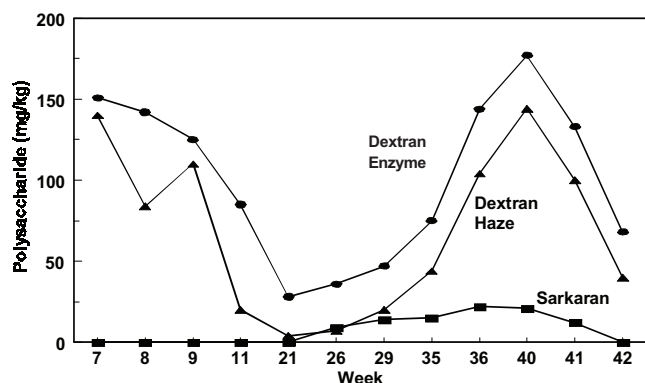


Figure 1. Seasonal trend in polysaccharides for NB refined sugar (1999).

enzymic dextran assay for refined sugar ($r^2 = 0.91$, $n = 12$ and slope = 0.93). The seasonal trend is shown in Figure 1, where it can also be seen that sarkaran was present from September onwards.

● *DAC extracts*

During a recent cane deterioration trial, sarkaran was detected in delayed burnt cut cane. About 250 mg/kg Bx was recorded after a fairly long induction period of two to three weeks (Barker, 2000).

● *General comments*

Because of the nature of polysaccharides, different methods will give different results. The indiscriminate application and reporting of polysaccharide results needs to be avoided. It is thus necessary to consider the reason for the measurement and the ultimate use to which it will be put. Furthermore, the necessary analytical precautions associated with a particular product must be applied and the method used should be reported together with the result.

The identity of sarkaran has only been based on the similarity of its behaviour to that reported previously *i.e.* that pullulanase yielded maltotriose and maltotetraose. Nevertheless, the compound is a distinct polysaccharide.

The procedure is also directly applicable to other polysaccharides where an enzyme is available which gives a characteristic product in quantitative yield. A similar procedure has been used to measure pectin in fruit products and preserving sugar.

Conclusions

An enzymic assay has been used to measure dextran and sarkaran in a variety of sugar products. Analytical characteristics of linearity, repeatability and reproducibility were good. The analyses are not affected by the presence of other polysaccharides. The techniques are, however, not suitable for rapid, routine applications.

The haze method was satisfactory for measuring high M Wt dextrans in both raw and refined sugars. The dextran observed in refined sugar was essentially high M Wt. The AOAC procedure does not correlate with either the haze or enzymic methods for dextran and appears to be of little value for predicting processing problems.

Mixed juice, VHP sugar and (in one case) refined sugar samples from three geographic regions gave typical, but different, polysaccharide distributions. Although the products are not matched they are representative of production during the period reviewed. It has been demonstrated that during the 1999 season high levels of sarkaran were evident at the Midlands mills in particular. This corresponded with the mills experiencing extremely viscous massecuite and molasses. Sarkaran was observed throughout the factory (juice, syrup, molasses and sugar) and persisted into the refined sugar towards the end of the season.

The presence of sarkaran in VHP sugar produced during 1997 suggests that the current observations are not unique, and

that the occurrence of sarkaran is considerably more widespread than has been realised. In addition, after a lengthy induction period, sarkaran was observed in burnt cane subjected to post-harvest delay.

Of considerable interest are the very distinct distributions of types of polysaccharides - it is not known whether these effects are varietal, climatic, environmental, maturity or disease related. Some of these avenues will form part of an on-going investigation into establishing the origin of sarkaran.

Acknowledgements

The assistance of Jean Thelemaque with the enzyme assays is gratefully acknowledged. The staff of the Analytical Division at the SMRI are thanked for the empirical analyses. James McClean of SK Chem Trade Services (Pty) Ltd generously supplied the dextranase, while the pullulanase was supplied by Enzymes SA (Pty) Ltd. The South African Sugar Terminal is thanked for obtaining the raw sugar samples. Finally thanks are due to the management of UC for involving the SMRI when they noticed extremely high viscosities in their factory and thus rekindled an interest in sarkaran.

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¹ The mentioning of a trade name or trademark does not infer a preference for a particular product or brand.

APPENDIX 1

Method for dextran and sarkaran in sugar products

Principle: Polysaccharides are enriched and isolated from sugar products by precipitation with ethanol (80% for total polysaccharides and 50% for high MWt polysaccharides). The precipitate is washed with ethanol to remove sugar and the concentrated polysaccharide material is then dissolved in water and an internal standard (e.g. raffinose) may be added. The dextran component is enzymically hydrolysed (to completion) using dextranase (sourced from *Chaetomium gracile*) and the major product (iso-maltose) is measured using isocratic HPAEC. The calibration is prepared by concurrent hydrolysis of standard dextran with added internal standard if preferred. The sarkaran component is enzymically hydrolysed using pullulanase and the products (maltotriose and maltotetraose) are measured using gradient HPAEC. The calibration is prepared by concurrent hydrolysis of standard pullulan.

Calibration:

- *Internal standard (raffinose):* The use of an internal standard is generally not necessary, but raffinose pentahydrate (0.06%) is suitable when using internal standardisation.
- *Stock dextran solution:* Weigh about 0.20 g Dextran T-500 (Pharmacia¹), after correcting for the moisture content. Dissolve with warming, if necessary, and dilute to 200 ml. Keep refrigerated and replace at least weekly. Prepare the calibration standards as indicated and dilute to 100 ml. If using internal standardisation, add 5.0 ml of internal standard before making to volume.

Standard	D-1	D-2	D-3	D-4	D-5	D-6
Dextran (ml stock)	0.2	0.5	1.0	2.5	5.0	10.0

- *Stock pullulan solution:* Accurately weigh about 0.25 g Pullulan (Sigma), after correcting for the moisture content. Dissolve with warming, if necessary, and dilute to 100 ml. Keep refrigerated and replace at least weekly. Prepare a working solution by diluting 10 ml of this stock solution to 100 ml. Prepare the calibration standards as indicated and dilute to 100 ml.

Standard	P-1	P-2	P-3	P-4	P-5	P-6
Pullulan (ml stock)	0.5	1.0	2.0	5.0	7.5	10.0

Isolation of polysaccharide (HMWt + LMWt):

- *Sugar:* (40 g, containing less than 2 500 ppm total or 1 250 ppm HMWt dextran) is dissolved in distilled water and diluted to 100 ml in a volumetric flask. Aliquots (10.0 ml for total or 20.0 ml for H MWt dextran) are transferred to centrifuge tubes (ca. 35 x 110 mm - capacity about 75 ml). Absolute ethanol (40 ml for total or 20 ml for H MWt) and 1.0 ml trichloroacetic acid (TCA) (10%) is added with stirring, followed by 0.2 g Celite 577. The TCA solution may be kept for two weeks, stored under refrigeration in a dark bottle. The samples are left to stand for about one hour. Centrifuge. Wash 5 times with 5 to 10 ml 80% ethanol for total, or twice with 5 to 10 ml 50% ethanol followed by 3 times with 5 to 10 ml 80% ethanol for H MWt dextran.
- *Other products:* Suitable sample sizes and dilutions for other products are indicated in Table 1.1. Sensitivity may be improved by increasing the sample size from which the polysaccharide is isolated. However, the convenience of carrying out the precipitation directly in the centrifuge tube is forfeited.

Table 1.1. Suggested dilutions for cane products.

Product	Sample (g or ml)	Diluted to (ml)	Aliquot (ml)	Ethanol added (ml)	Diluted to (ml)
DAC	25	-	25	100	50
MJ	10	-	10	40	50
Syrup	50	100	10	40	100
C-Molasses	10	100	10	40	100
Sugar - Raw/VHP - total	40	100	10	40	100
Sugar - Raw/VHP - HMWt	40	100	20	20	100
Refined sugar	40	100	10	40	50

Note: 20 ml aliquots are taken from the final dilution for hydrolysis and the volume after hydrolysis is adjusted to 25 ml. For samples with high polysaccharide concentrations smaller aliquots may be used for hydrolysis. For low levels of polysaccharide larger samples may be used for the isolation. If using internal standardisation, adjust the amount added according to the dilution volume.

Preparation of polysaccharide solution:

- **Sugar:** Dissolve the precipitate from the sugar in hot distilled water. If using an internal standard add 5 ml of 0.06% solution of raffinose pentahydrate and quantitatively transfer to 100 ml volumetric flasks. Do not allow the precipitate to stand for more than 1 hour before dissolving. Make to the mark and centrifuge to remove the filter aid. Transfer two 20.0 ml aliquots of supernatant to 50 ml beakers (diameter 40 mm). Use one set for dextran and the other for sarkaran.
- **Other products:** Suggested dilutions for cane products are given in Table 1.1.
- **Standards:** Transfer 20.0 ml aliquots of the standard solutions (D-1 to D-5) and (P-1 to P-6) to 50 ml beakers. Include a blank - 20 ml of water (or suitably diluted internal standard).

Hydrolysis of polysaccharides:

- **Enzyme activity:** A check needs to be carried out periodically to ensure that the following reaction conditions are adequate. Complete hydrolysis is necessary for these analytical applications. Hence the manufacturer's guidelines for measuring activity (generally in terms of reducing power) are meaningless in this context. The nominal activities are used as a starting point.
- **Preparation of dextran hydrolysate:** Add 1.0 ml diluted dextranase (Genencor Dextranex L- 4000, diluted 5 ml to 50 ml) to one aliquot, cover with a Petrie dish lid and incubate at 44°C overnight (16 to 18 hours). A set of dextran calibration standards must be included with each incubation to allow for variations, since the marker sugar (isomaltose) is generated from dextran.
- **Preparation of sarkaran hydrolysate:** Add 1.0 ml diluted pullulanase (Novo Promozyme, diluted 0.75 ml to 50 ml) to the other aliquot, cover with Petrie dish lid and incubate at 55°C for 60 minutes. A set of pullulan calibration standards must be included with each incubation to allow for variations, since the marker sugar (maltotriose) is generated from pullulan.

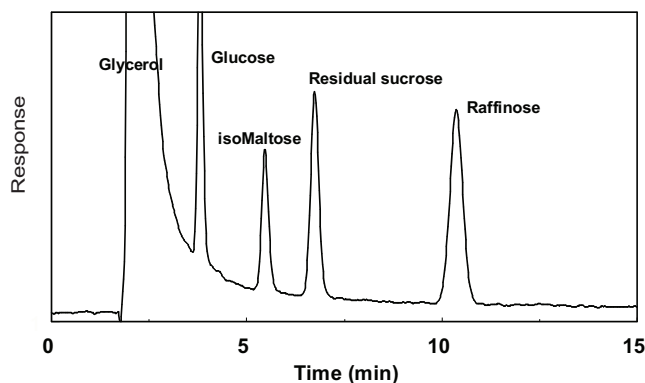


Figure 1.1. Chromatogram of isolated polysaccharide after dextranase hydrolysis. Refined sugar showing dextran product - isomaltose. Optional internal standard (raffinose) included.

The solutions are removed from the incubator and boiled (2 minutes) to inactivate the enzyme. Transfer to 25 ml volumetric flasks and make to volume. Filter (0.45 µm membrane).

Analytical conditions - HPAEC: The equipment and necessary precautions have been described earlier (Schäffler et al., 1996).

- **Dextran:** Sodium hydroxide (250 mM) is prepared as described previously by adding 13 ml NaOH (50%) per litre of solvent. The reference cell is filled with 250 mM NaOH. Cell settings have been defined previously (Schäffler et al., 1996). A range of 2 µA or 3 KnA is suitable when using the ESA CouloChem II or Dionex PAD controllers respectively. Flow rate is 0.8 ml/min. Injection volume is 20 µl. A typical separation is shown in Figure 1.1.
- **Sarkaran:** Sodium hydroxide (250 mM) (solvent A) and sodium hydroxide (250 mM)-sodium acetate (500 mM) (solvent B) are used to generate the following solvent gradient at a flow rate of 1ml/min:

Time (min)	Solvent A (%)	Solvent B (%)
0	95	5
20	0	100
21	95	5
30	95	5

The reference cell is filled with solvent B. Injection volume is 20 µl. A typical separation is shown in Figure 1.2.

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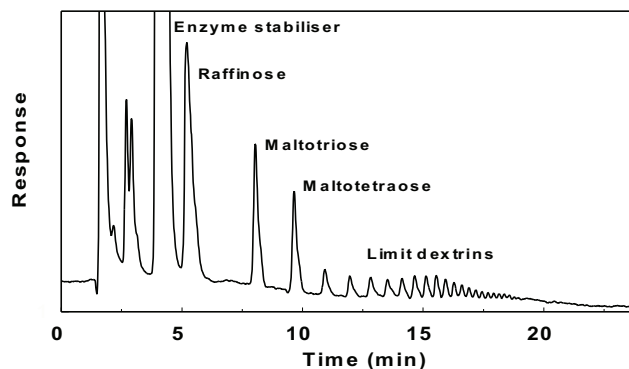


Figure 1.2. Chromatogram of isolated polysaccharide after pullulanase hydrolysis. Mixed juice with high starch content showing limit dextrans as well as sarkaran products - maltotriose and maltotetraose. Optional internal standard (raffinose) included.

APPENDIX 2

Table 2.1. Repeatability and reproducibility data for VHP sugar.

Sample	Dextran (mg/kg)				Sarkaran (mg/kg)			
	Day 1		Day 2		Day 1		Day 2	
	Repl 1	Repl 2	Repl 1	Repl 2	Repl 1	Repl 2	Repl 1	Repl 2
A	39	51	39	47	0	0	0	0
B	134	114	153	92	39	29	60	38
C	188	197	172	157	16	25	20	25
D	311	311	281	288	64	55	56	53
E	558	554	533	519	72	73	56	64
F	980	972	844	874	60	64	63	42

APPENDIX 3

Table 3.1. Dextran (enzymic) for weekly MJ composites (mg/kg Bx)

Mill	Week										
	24	26	27	28	31	33	34	35	36	37	39
ML		115	155	110	85			340	155	155	
KM1		70	90	110	80			290	95	110	
KM2		90	110	90	85			265	110	105	
AK		190	420	240	200			690	385	280	
NB	255	200	230	290	320	2 020	725	1 225	1 320		5 085
UC	115	80	105	105	265	1 055	430	1 525	875	200	4 595
SZ1	385	380	595	565	500	1 055	1 240	3 605	660	690	2 620
SZ2	600	740	1 070	735	720	2 080	3 275	3 650	1 600	1 060	2 735

Table 3.2. Sarkaran (enzymic) for weekly MJ composites (mg/kg Bx)

Mill	Week					
	24	31	33	34	36	39
ML		200			150	
KM1		255			170	
KM2		175			125	
AK		320			275	
NB	365	2 435	1 375	2 250	2 145	2 310
UC	105	1 445	1 075	1 820	1 980	1 230
SZ1	370	285	515	320	1 095	510
SZ2	245	360	505	540	595	450