

ARE MANNITOL AND LACTIC ACID INDICATORS OF SUGARCANE DETERIORATION IN A SOUTH AFRICAN CONTEXT?

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Abstract

Deteriorated sugarcane can lead to processing difficulties in the factory and to high levels of unwanted compounds in the raw sugar, with some extending to refined sugar. A high level of dextran is one of the more serious processing problems in a sugar factory that is associated with deteriorated cane. The development of a rapid test to measure an indicator of the 'amount of deterioration' could be useful to determine whether a load of cane can be processed economically or not. Ethanol has been used as an indicator in the South African industry for many years. Indicators used in other sugarcane industries include dextran itself (monoclonal antibody method) and mannitol. This study describes an investigation of the possible use of mannitol or lactic acid as indicators of cane deterioration as measured by dextran. It further investigates the use of a rapid enzymatic method for mannitol analysis as an indicator of cane deterioration for use at individual mills.

Keywords: sugarcane, deterioration, mannitol, dextran, lactic acid

Introduction

It is well documented that deteriorated cane can lead to processing difficulties in the factory, including effects on viscosity, clarification processes and polarimetry (Atkins and McCowage, 1984). Some of these unwanted deterioration products are carried through to the raw and refined sugars, causing processing difficulties in other processing industries. Much of the deterioration is caused by microorganisms such as bacteria, yeasts and fungi. Many compounds are produced during the deterioration period, including metabolites such as lactic acid and mannitol, and final fermentation products such as dextran and ethanol. One of the more serious processing problems in a sugar factory associated with deteriorated cane is high levels of dextran. The dextran is produced by the bacterium *Leuconostoc mesenteroides*. Billed cane and burnt cane experience higher deterioration rates than green harvested whole stick cane, and consequently high dextran levels, especially during periods of hot and wet weather, as a result of exposure of the inside of the stalks to *L. mesenteroides*.

It is possible to hydrolyse (break down) dextran into smaller molecules using dextranase enzyme, and this is practised in the Australian and United States milling industries during limited periods of processing deteriorated cane. Dextranase has been added to first expressed juice, into the last effect evaporator or syrup tank (Cuddihy *et al.*, 2000). Laboratory trials at the Sugar Milling Research Institute (SMRI) have shown that dextranase treatment of diffuser mixed juices is unsuitable because of the high temperature and low Brix conditions encountered in this part of the process that render the enzyme inactive (Morel du Boil and Wiense, 2002). The trials also showed that it was technically feasible to reduce high levels of

dextran in evaporator syrup. However, high enzyme dosages are necessary to compensate for Brix and temperature inactivation, and this will be uneconomical.

Throughout the world, studies have focused on developing rapid tests to measure the “amount of deterioration” so that the results can be used to determine whether a load of cane can be processed economically or not. Tests developed include the monoclonal antibody method (to measure dextran directly; Day and Plhak, 1998) or more recently the use of mannitol as an indicator of deterioration (under US conditions; Eggleston *et al.*, 2008). The SMRI has shown a good correlation between lactic acid and dextran (under limited South African conditions; Morel du Boil, 2005).

Previous studies

Although increased dextran, lactic acid, mannitol or ethanol concentrations are all indicative of cane deterioration, they are not necessarily interrelated or reliable deterioration indicators, as different mechanisms operate under different environmental conditions.

The synthesis of dextran by the dextransucrase elaborated by *Leuconostoc* species using sucrose as substrate gives rise to several by-products. Part of the sucrose is converted outside of the cell into dextran and fructose. The rest (with the fructose) enters the bacterial cell, where it is converted to other products including D-lactate, acetate, ethanol, mannitol and CO₂. The extent of this heterolactic fermentation is influenced by conditions of temperature, pH, incubation time, substrate concentration and oxygen availability. Most of these products can arise from other mechanisms as well. Their potential usefulness as predictors of processing difficulties lies in the fact that they can generally be measured more easily and rapidly than can dextran. Provided that dextran formation from sucrose is the dominant reaction, lactic acid, mannitol or ethanol can be used as indicators of deterioration. In South Africa, ethanol in Direct Analysis of Cane extracts as an indicator of cane delay has been studied by Lionnet and Pillay (1987, 1988) and has been used by a number of mills to help minimise burn-to-cut-to-harvest delays. In the United States, Saska (2002) reported that lactic acid or ethanol were more sensitive indicators of the early stages of deterioration than dextran. More recently, mannitol formation as an indication of *Leuconostoc* activity in Louisiana freeze-damaged cane has been studied by Eggleston and co-workers (Eggleston, 2002, 2003; Eggleston and Legendre, 2003; Eggleston *et al.*, 2004).

In recent work by Eggleston *et al.* (2008), the authors found a polynomial relationship between mannitol and dextran (as measured by the haze method) in factory crusher juice across *one season for a single Louisiana factory* (Figure 1). It should be noted that the haze dextran values reported are extremely high and well outside the range of typical South African values. Morel du Boil (2005) had previously found a linear correlation between lactic acid and total dextran (High Performance Anion Exchange Chromatography (HPAEC) method). The samples were taken over a four year period (2000 to 2003) and were sourced primarily from factories on the South Coast region of KwaZulu-Natal (Figure 2). It should be noted that the relationship may not be applicable in a wider context.

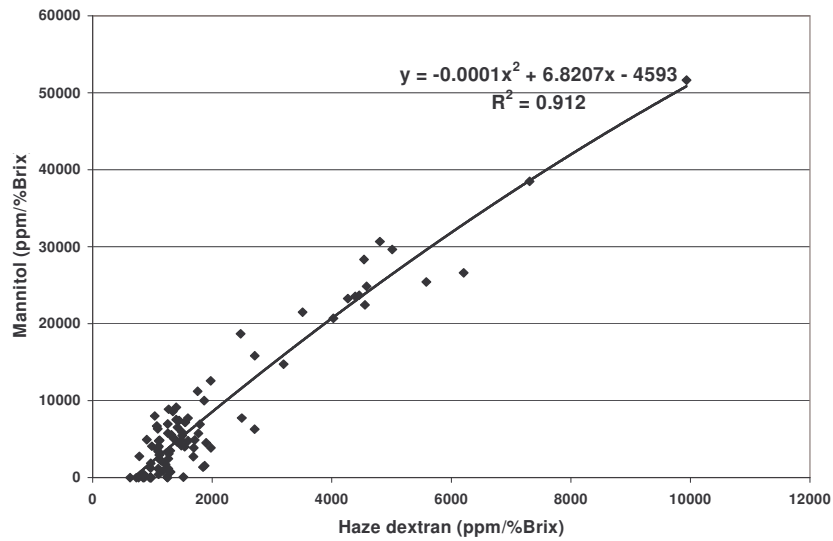


Figure 1. Relationship between mannitol and haze dextran in press and crusher juices collected across the 2004 processing season at a Louisiana, US factory (N=188). (From Eggleston *et al.*, 2008.)
(Note that ppm%/Bx is equivalent to mg/kg Bx.)

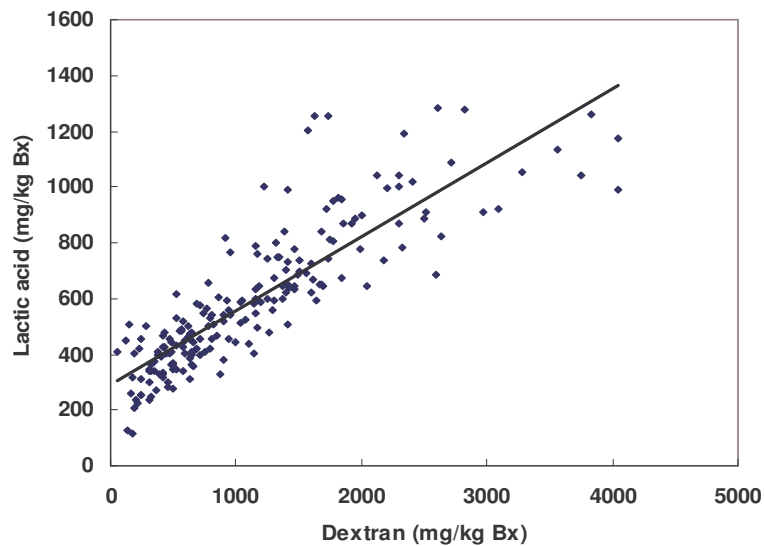


Figure 2. Scatter plot for lactic acid (chromatographic method) in mixed juice against dextran for 2000 to 2003 seasons in South Africa (N=196). (From Morel du Boil, 2005.)

The current paper describes the correlations found between mannitol or lactic acid as indicators of cane deterioration as measured by dextran. It further investigates the possible use of a rapid enzymatic method for mannitol analysis as an indicator for cane deterioration for use at individual mills.

Experimental

Methods for initial study of mannitol, lactic acid and cane deterioration (dextran)

A sub-sample of the weekly Cane Testing Service mixed juice (MJ) composite samples received by the SMRI for factory control and cane payment purposes from four mills from South African Sugar Association (SASA) weeks 27 to 42 (August to December 2007) were analysed. Existing SMRI chromatographic methods were used to measure mannitol (HPAEC; Anon, 1997), lactic acid (Headspace Gas Chromatography; Anon, 1997), and total and high molecular weight (HMW) dextran (Morel du Boil, 2000). Note that HMW dextran has been shown previously to correlate with dextran measured by the haze method (Morel du Boil, 2005) (the method used by the Terminal and mill laboratories; Anon, 2004). All results are expressed as mg/kg Bx.

Evaluation of an enzymatic mannitol method

The spectrophotometric enzymatic method used for the determination of D-mannitol by mannitol dehydrogenase is based on the Megazyme method K-Manol 01/05 (Anon, 2005). Mannitol dehydrogenase (MDH) was obtained from Megazyme International, Ireland, and D-mannitol, Bovine Serum Albumin (BSA) and Nicotinamide-Adenine Dinucleotide (NAD) from Sigma-Aldrich. Synthetic mixed juice samples were prepared from Analar sucrose, glucose and fructose. A sample of frozen, preservative-free mixed juice (500 ml in 20 ml sachets; Anon, 1985) was obtained from Eston mill. Solution absorbance values were read on a Perkin-Elmer Lambda 25 spectrophotometer. All results are expressed as mg/kg Bx.

Results

A summary of the analytical results obtained for the four mills from the initial study is shown in Table 1. The range of weeks chosen for sampling encompassed both normal levels of the analytes (weeks 27 to 32) and periods when elevated analyte levels can occur (weeks 32 onwards).

Table 1. Summary of analytical results from four mills for South African Sugar Association weeks 27 to 42.

Mill		Total dextran (mg/kg Bx)	HMW dextran (mg/kg Bx)	Mannitol (mg/kg Bx)	Lactic (mg/kg Bx)
A	min	330	85	150	300
	max	2780	2010	650	825
B	min	175	70	520	395
	max	1730	500	1675	950
C	min	135	90	605	420
	max	4345	2430	3105	1625
D	min	140	45	335	215
	max	3730	2545	1860	805

Mannitol as an indictor

Acceptable linear correlations between mannitol and HMW dextran were found ($R^2 \approx 0.82$) at individual factories (Table 2 and Figure 3). However, the correlation on the combined data was poor ($R^2 \approx 0.38$). This indicates that, whilst localised deterioration indicators may be applicable, it is not possible to apply them globally.

Table 2. Regression data for high molecular weight (HMW) dextran (HPAEC*) and mannitol.

Mill	Constant	Slope	R ²	N
A	198.65	0.2895	0.82	13
B	371.47	1.7190	0.84	10
C	944.73	0.6856	0.80	13
D	536.74	0.5729	0.83	10
Overall	549.18	0.5885	0.38	46

*High performance anion exchange chromatography

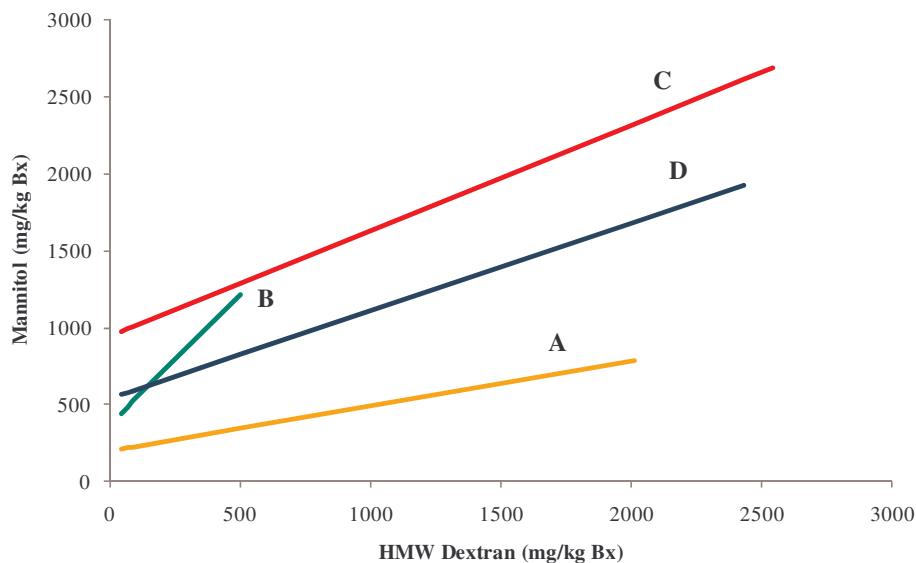


Figure 1. Regression lines for individual mills for high molecular weight (HMW) dextran in mixed juice against mannitol (chromatographic method).

A similar trend was found for total dextran and mannitol (Table 3 and Figure 4).

Table 3. Regression data for total dextran (HPAEC*) and mannitol.

Mill	Constant	Slope	R ²	N
A	148.3	0.207	0.74	13
B	427.2	0.770	0.81	10
C	887.5	0.442	0.82	13
D	459.2	0.423	0.83	10
Overall	424.1	0.480	0.53	46

*High performance anion exchange chromatography

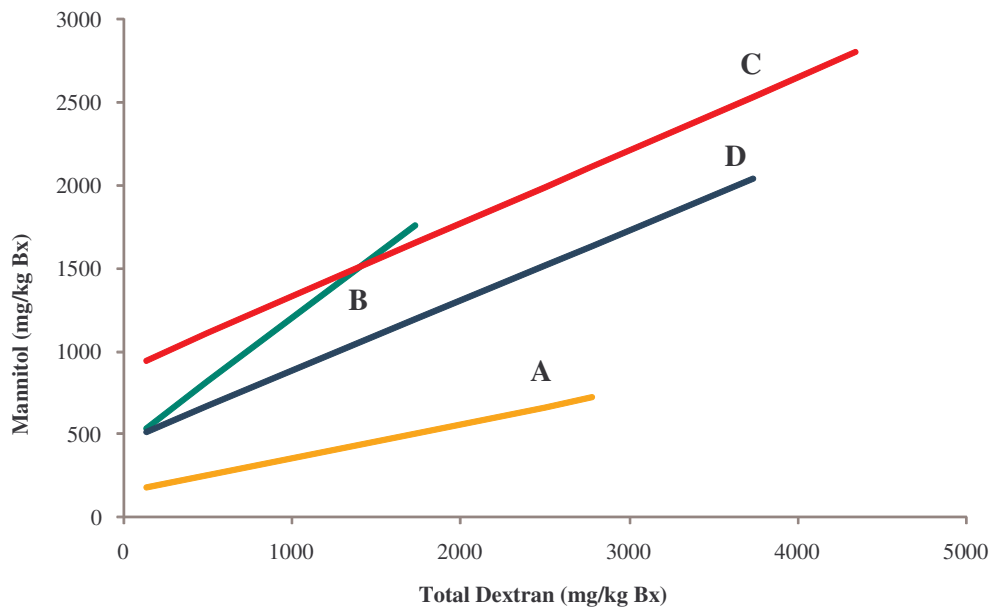


Figure 2. Regression lines for individual mills for total dextran in mixed juice against mannitol (chromatographic method).

The maximum mannitol concentration measured in this study was 3105 mg/kg Bx (corresponding to 4345 mg total dextran/kg Bx); the next highest mannitol concentration was 2365 mg/kg Bx (corresponding to 2720 mg total or 1585 mg HMW dextran/kg Bx). In South Africa this level of dextran is considered extremely high, yet falls into the lowest 15% of the dextran range covered in the Louisiana study (Figure 1). Similarly, mannitol at 2365 mg/kg Bx lies in the lowest 4% of the range covered in Figure 1. Under South African conditions, the ratio of mannitol to HMW dextran is of the order of two to four times less.

Under standard conditions mannitol may have application as a deterioration indicator or dextran predictor on a regional basis, but it is not necessarily true that the relationship will be similar from mill to mill or from season to season. Thus, it is probably unlikely that a fixed cut-off limit could be prescribed at the factory gate in advance. The relationship shown between mannitol and dextran is historical. There is no guarantee that the same relationship will hold in the following season, thus limiting its use as a deterioration indicator or dextran predictor. Hence, as with all previously suggested deterioration indicators, there are severe limitations on its suitability unless deterioration is extensive.

Lactic acid as an indicator

In this study lactic acid did not correlate well with dextran other than at one mill (mill C) (Table 4).

Table 4. Regression data for total dextran (HPAEC*) and lactic acid.

Mill	Constant	Slope	R ²	N
A	369.3	0.135	0.35	15
B	574.4	0.165	0.14	15
C	445.5	0.188	0.86	15
D	364.5	0.102	0.33	15
Overall	429.2	0.169	0.43	60

*High performance anion exchange chromatography

Mixed juice samples from three of the four factories surveyed, contained more mannitol than lactic acid. However, samples from mill A contained, on average, 150 mg/kg Bx more lactic acid than mannitol. The largest difference was 470 mg/kg Bx. The chromatographic method used to measure lactic acid includes both D- and L-lactic acid and so is not specific for *Leuconostoc* deterioration (where D-lactate is produced). However, the difference in behaviour from factory to factory serves to highlight that many different degradation pathways are operational. It should also be noted that different *Leuconostoc* strains may give different metabolite ratios and other bacterial species could interfere with some of these indicators, e.g. *Lactobacillus* can give rise to lactic acid.

In the case of mill C, where dextran concentrations approached 2 500 to 3 000 mg/kg Bx from Week 33 onwards (Figure 5), both lactic acid (R²=0.858) and mannitol (R²=0.821) were equally suitable predictors of dextran (Figures 6 and 7).

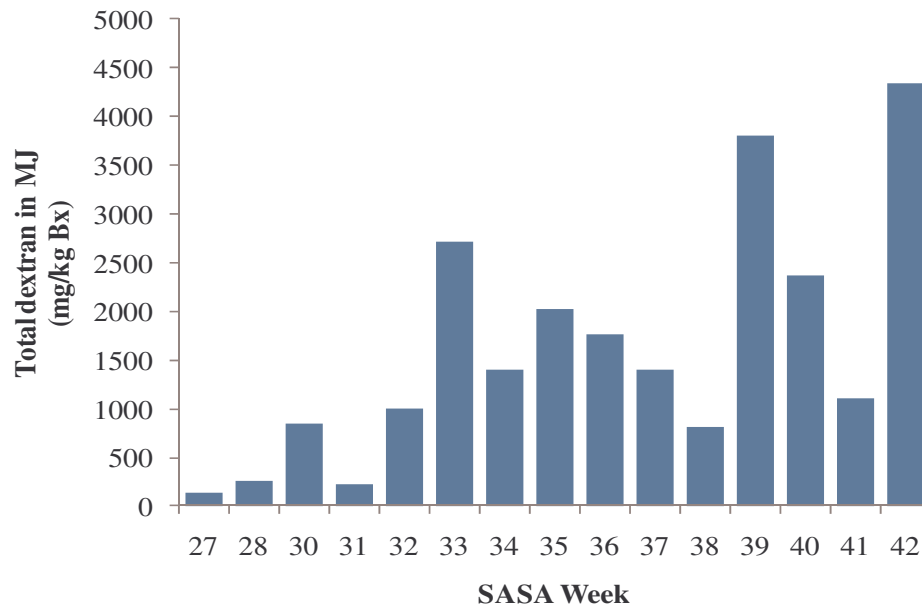


Figure 3. Seasonal variation in MJ total dextran at mill C for 2007.

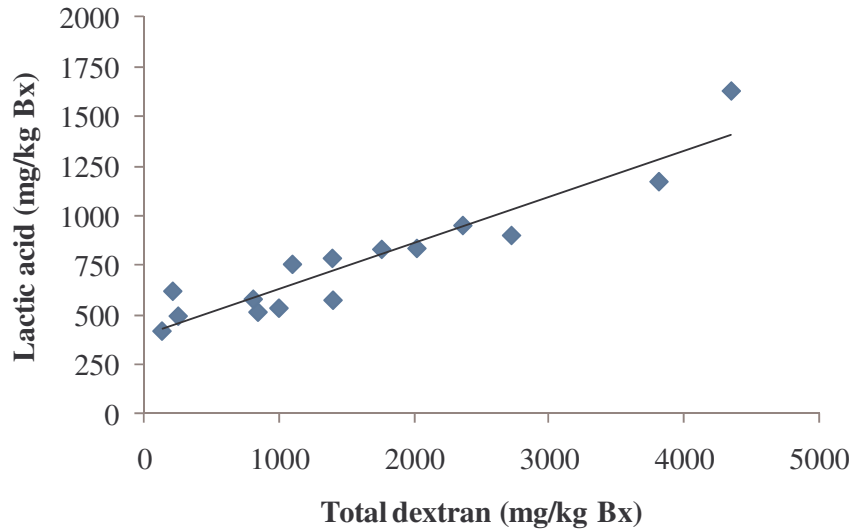


Figure 4. Relationship between total dextran and lactic acid for mill C, 2007 season.

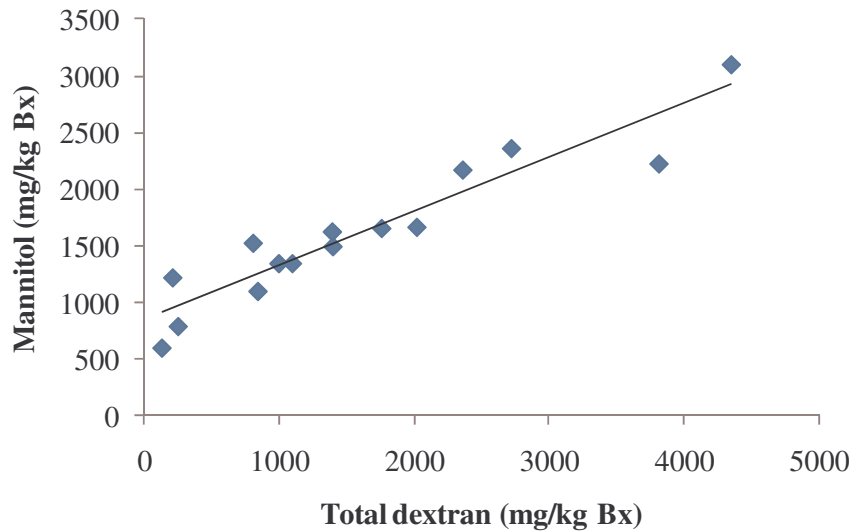


Figure 5. Relationship between total dextran and mannitol for mill C, 2007 season.

During this study, HMW dextran (which is similar to haze dextran) did not exceed 2500 mg/kg Bx, while total HPAEC dextran did not exceed 4300 mg/kg Bx, which is low in comparison to the work reported by Eggleston *et al.* (2008).

Evaluation of a rapid enzymatic method for mannitol analysis

All mannitol values in the initial study were determined using HPAEC. The capital and operating costs and maintenance of this equipment is prohibitive for a mill laboratory environment. Therefore, if the measurement of mannitol concentration in juices were to be considered for use as an indicator for deterioration, a simple, rapid method suitable for use in

mill laboratories would be required. A rapid spectrophometric enzymatic mannitol assay was therefore investigated. The assay was found to be linear over a range of 5-75 μg of D-mannitol (equivalent to 0.05-0.75 g/litre or 50-750 ppm (m/v)) in solution (Figure 8).

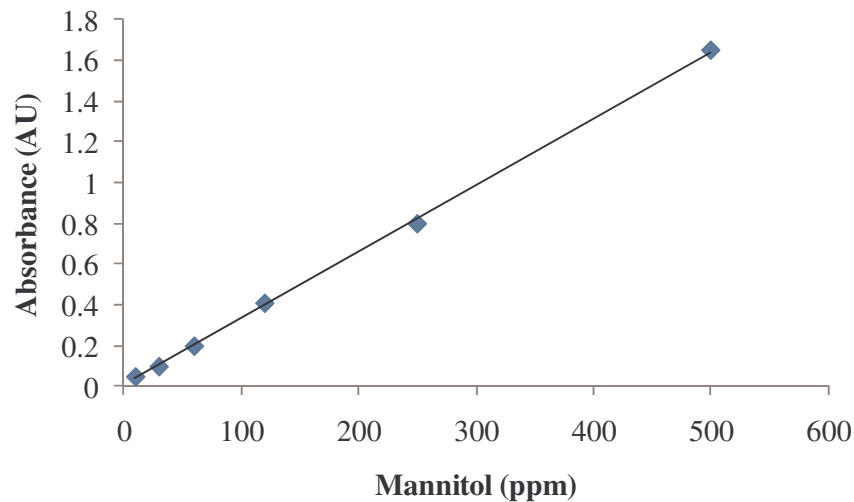


Figure 6. Linearity of the enzymatic mannitol method.

The recovery of the method was determined by preparing recovery samples at a series of concentrations representing typical mannitol levels found in South African MJ (15-250 ppm in solution [\sim 150 to 2500 mg/kg Bx]). These samples were analysed and showed good recoveries for mannitol in water (Table 5).

Table 5. Mannitol prediction from prepared samples of mannitol.

Original concentration (ppm in solution)	Calculated concentration (ppm in solution)	Recovery (%)
15	16	107
30	31	103
60	63	105
120	123	103
240	240	100

As the method was being evaluated for use in MJ samples, possible interference by sucrose, glucose and fructose at the levels typically found in MJ was checked by adding sucrose (10%), fructose and glucose (0.5% each) to solutions containing only mannitol (Table 6).

Table 6. Mannitol prediction from solutions containing sucrose (S), glucose (G) and fructose (F).

Matrix	Original mannitol concentration (ppm in solution)	Calculated mannitol concentration (ppm in solution)	Recovery (%)
No FGS	60	63	105
	120	119	99
FGS	60	26	43
	120	48	40
Sucrose only	120	110	92
Glucose only	120	116	97
Fructose only	120	44	37

The presence of a mixture of sucrose, glucose and fructose in the mannitol solution resulted in the method under-estimating the amount of mannitol in solution. Addition of individual sugars (sucrose, glucose and fructose) to the mannitol solution showed that the low predictions were due to the presence of fructose. This lowering effect can be explained when studying the principle of the enzymatic method (equation 1):



D-mannitol is oxidized by nicotinamide-adenine dinucleotide (NAD⁺) to D-fructose in the presence of mannitol dehydrogenase (ManDH) with the formation of reduced nicotinamide-adenine dinucleotide (NADH). The NADH that is produced is measured spectrophotometrically as an indication of the quantity of mannitol present in the solution. This is a reversible reaction, as indicated by the reversible arrows. If excess D-fructose is present (relative to the mannitol), the ManDH will produce D-mannitol until equilibrium is attained (Edmundowicz and Wriston, 1963; Lee *et al.*, 2003). The amount of NADH produced will therefore be less and result in a lower absorbance value. In the case of the synthetic mixed juice, the amount of fructose present is far in excess of mannitol (5000 ppm fructose versus 120 ppm mannitol in Table 6) and the reaction will not go to completion and therefore will underestimate the concentration of mannitol – in this case by more than 50%. Based on these results, no analysis of mixed juice was undertaken.

This mannitol method has been reported to predict sugarcane deterioration at factories (Eggleston and Harper, 2006; Eggleston *et al.*, 2008). These reports show that the method was developed and checked under Louisiana conditions. Interference from sucrose, glucose and fructose was checked at mannitol levels of 1000 to 2000 ppm *in solution* (approximately 7000 to 14 000 mg/kg Bx). This should be compared with normal South African conditions of 20 to 50 ppm in solution and 150 to 250 ppm in solution under adverse conditions (Table 1). The minimum mannitol reported by Eggleston was 7500 mg/kg Bx, with values routinely measuring 25 000 to 50 000 mg/kg Bx at one mill! Under these conditions the ratio of mannitol to fructose is so great that the reaction will proceed to the right in equation 1 and the

mannitol can be easily quantified. Fructose interference at these elevated mannitol levels using the enzymatic method was checked by preparing synthetic solutions with high levels of mannitol and levels of fructose considered normal for South Africa (Table 7).

Table 7. Mannitol recovery from solutions at high concentration.

Original mannitol concentration (ppm)	No added fructose		0.5% added fructose	
	Calculated mannitol concentration (ppm)	Recovery (%)	Calculated mannitol concentration (ppm)	Recovery (%)
1250	1220	98	1030	82
2500	2260	91	2240	89
5000	4605	92	5000	100
10000	9410	94	9360	94

Recovery at these very high levels is acceptable and agrees with the findings of Eggleston, *et al.* (2008). However, these levels are considerably higher than the elevated levels found under South African conditions.

Conclusions

Weekly MJ samples from four mills were used to determine whether correlations existed between mannitol or lactic acid as indicators of cane deterioration as measured by dextran. Using HPAEC, acceptable correlations were found between mannitol and dextran at individual factories but not on an industry wide basis. Lactic acid as an indicator gave an acceptable correlation at only one mill.

The possible use of a simple, rapid enzymatic method for the measurement of mannitol to be used as the basis for a measure of cane deterioration has been evaluated. The method was found to be linear and gave good recoveries when used with standard solutions. However, fructose was found to interfere with the mannitol measurements at the levels of mannitol encountered under South African conditions, underestimating by more than 50%. Using conditions under which the method was developed, *viz.* mannitol concentrations 10 to 20 times greater than local concentrations, the method gave acceptable results.

Based on the results presented here it can be seen that, although mannitol and lactic acid may be used as possible indicators of cane deterioration, the practical application of a rapid enzymatic test is problematic due to the levels of fructose relative to the mannitol, and the use of such a test is not recommended for South African conditions.

Acknowledgement

The authors wish to thank South African Sugar Millers Ltd for financial support for this study.

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