



Analytical Methods

Viability of an enzymatic mannitol method to predict sugarcane deterioration at factories

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ABSTRACT

The delivery of consignments of deteriorated sugarcane to factories can detrimentally affect multiple process units, and even lead to a factory shut-down. An enzymatic factory method was used to measure mannitol, a major degradation product of sugarcane *Leuconostoc* deterioration in the US, in press (consignment) and crusher juices collected across the 2004 processing season at a Louisiana factory. Weather conditions varied markedly across the season causing periods of the delivery of deteriorated sugarcane to the factory. A strong polynomial relationship existed between mannitol and haze dextran ($R^2 = 0.912$) in press and crusher juices. Mannitol concentrations were usually higher than haze and monoclonal antibody dextran concentrations, which indicates: (i) the usefulness and higher sensitivity of mannitol to better predict sugarcane deterioration from *Leuconostoc* and other bacteria than dextran, and (ii) the underestimation by sugar industry personnel of the relatively large amounts of mannitol present in deteriorated sugarcane that can affect processing. Greater than ~2500 ppm/%Brix mannitol in juice predicts downstream processing problems. The enzymatic method is quantitative and could be used in a sugarcane payment formula. Approximately >300 ppm/%Brix haze dextran in raw sugar indicated that the majority of the crystals were elongated. Approximately >600 ppm/%Brix antibody dextran indicated when elongated crystals were predominant in the raw sugar. The enzymatic mannitol method underestimates mannitol in raw sugars.

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1. Introduction

The delivery of consignments of deteriorated sugarcane to factories can detrimentally affect multiple process units, and even lead to a factory shut-down. Currently, there is no validated, reliable, rapid, easy, and inexpensive method to measure sugarcane deterioration at the factory. This has meant that factory personnel have not been able to screen individual consignments of sugarcane and, thus, they do not know which consignments will detrimentally affect processing and are unable to reject unsuitable consignments. Furthermore, with the worldwide emphasis of delivering high quality sugarcane to the factory, sugarcane payment formulas incorporating a deterioration quality parameter may serve as a deterrent against the delivery of overly deteriorated sugarcane, improve processing, and encourage better sugarcane management.

The major (but not sole) contributor to sugarcane deterioration in the United States, particularly Louisiana where humid conditions prevail, is infection by *Leuconostoc* lactic acid bacteria.

Factors affecting infection are ambient temperature and humidity, level of rainfall and mud, length of sugarcane billet, degree of burning, billet damage, delays between burning and cutting and subsequent processing, and mill hygiene (Eggleston, Monge, & Montes, 2007a).

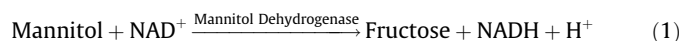
Previously, the sugar industry has considered dextran, a high viscosity glucopolysaccharide, as the major deterioration product of a *Leuconostoc* infection. High concentrations of dextran (>1000 ppm/%Brix) can reduce evaporation and crystallization rates, and the factory is penalized by refineries for excessive dextran in the raw sugar. Current methods to determine dextran, however, are either too long or complicated (ASI enzymatic method; Sarkar & Day, 1986), not specific enough, too expensive (antibody method; Rauh, Cuddihy, & Falgout, 2001), or too difficult in the interpretation of results (haze method; Clarke, Bergeron, & Cole, 1987). Furthermore, it is now known that mannitol, a sugar alcohol, is also a major degradation product of *Leuconostoc* sugarcane deterioration (Eggleston & Legendre, 2002; Eggleston & Harper, 2006; Eggleston, Legendre, & Tew, 2004), sugarbeet deterioration (Steinmetz, Buczy, & Bucholz, 1998; Thielecke, 2002) and the bacterial contamination of fuel ethanol produced from sugarcane (Eggleston, Basso, Amorim, De Lima Paulillo, & Basso,

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2007b). Mannitol is a more sensitive indicator of sugarcane deterioration than dextran and also produced by other *Lactobacillus* bacteria, although *Leuconostoc* is the greatest producer (Eggleston et al., 2007b). Mannitol can predict sucrose losses and dextran related problems such as viscosity and, to a lesser extent, filterability problems (Eggleston et al., 2004). Moreover, mannitol can occur in large amounts in factory syrups and massecuites processed from deteriorated sugarcane, does not degrade under processing conditions (Eggleston et al., 2004) and directly affects processing by reducing sugar recovery (Bliss, 1975) and evaporation rates (Eggleston & Harper, unpublished results).

Eggleston and Harper (2006) developed an enzymatic method to measure mannitol in sugarcane juices at the factory, because chromatography techniques are too sophisticated for use at the factory; furthermore, a high level of expertise is required by the operator (Eggleston et al., 2007b). The method utilizes mannitol dehydrogenase (MDH) to convert mannitol to fructose in the presence of co-enzyme NAD⁺. The NADH formed can be easily measured spectrophotometrically at 340 nm



The method is rapid (~7 min at room temperature and within 4 min if a 40° C water bath is used to incubate the juice), accurate, precise, highly specific for mannitol, and is not affected by the presence of sucrose, glucose, fructose, or dextran. It can be easily performed using existing equipment at the factory. The current cost per analysis of mannitol in a sugarcane load at the factory is only ~60 US cents, with the largest cost being NAD at 45 cents per analysis (Eggleston & Harper, 2006). However, for the mannitol enzymatic method to be a viable factory method, it needs to be tested on factory samples subjected to varying environmental and industrial conditions. This paper reports the viability of the method, compared to other dextran methods used sporadically by many sugarcane factories, across a sugarcane processing season in the US. The measurement of mannitol in both press (consignment) and crusher juices is reported, to observe its potential as a factory measurement to control and predict processing problems and its use in sugarcane payment formulas.

2. Materials and methods

2.1. Chemicals and enzymes

Mannitol dehydrogenase (EC 1.1.1.67) was purchased as a freeze-dried powder (8.45 U/mg dry weights) from Biocatalyst Ltd., Wales. All chemicals used were analytical grade.

2.2. Factory conditions

This study was conducted across the 2004 processing season at Iberia Sugar Cooperative sugarcane factory in New Iberia, Louisiana, USA. The season average factory processing and juice flow rates were 6490 metric tonnes (7154 short tons of cane)/day and 270.3 metric tonnes (298 short tons of cane)/hour, respectively. Approximately 10% non-billeted material (i.e., leaves, tops, and mud) entered the factory, with more at the beginning of the season when the sugarcane was shorter because of chemical ripener treatment. Approximately 85% of the sugarcane was billeted; ~70% of the billeted sugarcane was green (unburnt) and 30% burnt.

2.3. Factory sampling

Samples (120 ml) of press juices from different consignments of sugarcane were collected at the factory core laboratory. Press juices were obtained after a random core sample of a grower's

consignment of sugarcane was passed through a hydraulic press. Four drops of biocide (Bussan 881™, Buckman Labs.) were added to each juice to prevent further deterioration, and the samples were then placed in a –20° C freezer at the core laboratory. Every three to four weeks, the samples were collected and transported in dry ice to Dr. Eggleston's laboratory in New Orleans, where they were stored in a –60° C freezer until analyses. Crusher juices (120 ml) from the first tandem mill were also collected randomly across the season. The samples were biocide treated and stored the same as for the press juice samples. Raw sugar samples (~140 g) were collected as 24 h composites (6:00 a.m.–6:00 a.m.) from the factory every day. Randomly chosen raw sugars across the season were analyzed.

2.4. Weather conditions across the 2004 processing season

Average temperature and rainfall data for the New Iberia area across the 2004 processing season (9 October to 11 December) are illustrated in Fig. 1. From 17 November to 9 December there was an usual amount of rainfall with some sporadic warm days, that were favorable to the deterioration of sugarcane in the area.

2.5. Mannitol in press and crusher juices

The enzymatic factory method of Eggleston and Harper (2006) was used. The procedure was undertaken at room temperature (~25° C). Mannitol was expressed as ppm/%Brix or refractometric dry solids (rds).

2.6. Rapid haze dextran in press and crusher juices

Rapid haze dextran in juices was based on the modified method of Eggleston and Monge (2005). Termamyl™ (Novo, US, EC 3.1.1.1) amylase enzyme was added to juice to remove interfering starch then the juice was diluted. If the absorbance exceeded the higher limit of the calibration curve a greater dilution was undertaken. Dextran T2000™ was the standard and dextran was precipitated with 100% absolute ethanol. For raw sugars, ~14 g were first dissolved in 86 ml of de-ionized water and the Brix measured of the solution. No dilution was required to measure the rapid haze dextran content of the raw sugar solution because of the relatively low dextran levels compared to juices. Haze dextran was expressed as ppm/%Brix or rds.

2.7. Monoclonal antibody dextran in press and crusher juices

The Rapid Dextran Test or SucroTest™ (Midland, US) was used (Anon., 2003; Rauh et al., 2001) to measure antibody dextran in randomly chosen press and crusher juices (total 33) across the processing season. A conversion factor was calculated for each batch of antibody used. For raw sugars, the samples were first diluted to ~14 Brix before analysis. Antibody dextran was expressed as ppm/%Brix or rds.

2.8. Brix

The mean Brix of triplicate samples was measured using an Index Instruments TCR 15–30 temperature controlled refractometer accurate to ±0.01 Brix.

2.9. Raw sugar crystal shape

The raw sugar crystal shape was evaluated using an Olympus Mic-D digital microscope (Center Valley, US). At least two random sub-samples of each raw sugar were evaluated.

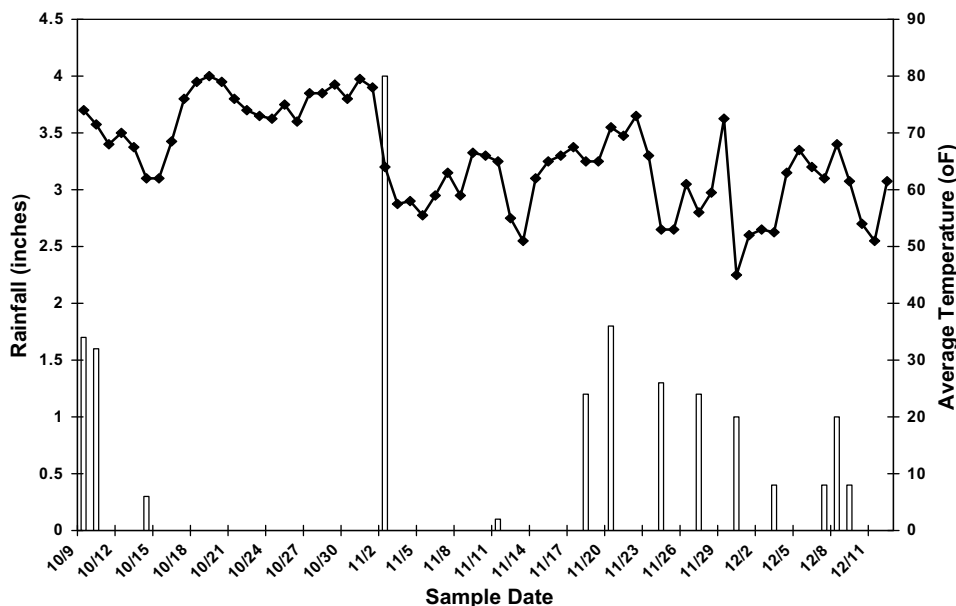


Fig. 1. Average rainfall and temperature data at the factory across the 2004 processing season.

3. Results and discussion

3.1. Mannitol and haze dextran in factory press and crusher juices

The enzymatic mannitol method of Eggleston and Harper (2006) was used to determine mannitol concentrations in 188 press and crusher juices across the 2004 processing season at a factory in Louisiana, US (Fig. 2). Each press juice represents an individual grower's consignment of sugarcane delivered to the factory, and analyses of press juices are undertaken at the factory for grower sugarcane payments. Each crusher juice from the first tandem mill represents a random mix of juices from multiple consignments that have been mixed, shredded, and passed through the first roller tandem mill in the factory. As numerous factories measure dextran in juice with a rapid haze method, this analysis was also undertaken and results shown in Fig. 2. The spikes in mannitol concentrations on 18 October, 2–3 November, and from 17 Novem-

ber to 4 December are mostly attributable to the occurrence of rainfall before or on these dates with a concomitant spike in temperature (Fig. 1). Mannitol clearly detected sugarcane deterioration much better than haze dextran from 7 November to 4 December (Fig. 2). Mannitol concentrations were generally higher than haze dextran concentrations, which agree with previous results (Eggleston et al., 2007b). This further highlights (i) the usefulness and higher sensitivity of mannitol to better predict sugarcane deterioration from *Leuconostoc* and other bacteria than dextran, and (ii) the underestimation by sugar industry personnel of the relatively large amounts of mannitol present in deteriorated sugarcane that can affect processing.

The relationship between mannitol and haze dextran in both press and crusher juices is further illustrated in Fig. 3. The measurement of haze dextran when no mannitol was detected can be explained by the nature of the haze dextran method. This method measures all haze forming material precipitated by absolute ethanol (i.e., medium molecular weight (MMW) starch if amylase is incorporated into the method, indigenous cane polysaccharide (Blake & Clarke, 1984) and other compounds that create "haze" such as bagasse microparticles) and, therefore, there is always a

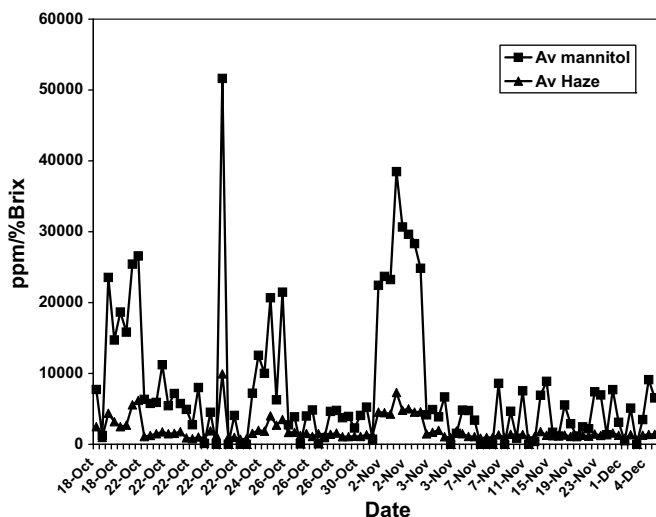


Fig. 2. Variations in mannitol and haze dextran in pressed and crusher juices across the 2004 processing season at a Louisiana sugarcane factory ($N = 188$).

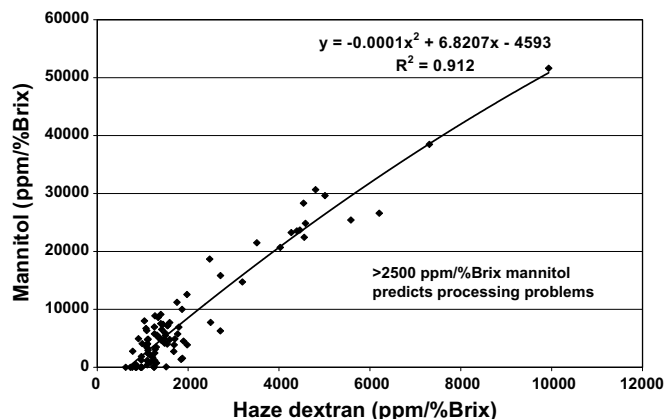


Fig. 3. Relationship between mannitol and haze dextran in press and crusher juices collected across the 2004 processing season at a Louisiana factory ($N = 188$).

background haze measurement. For example, in fresh sugarcane juice there is approximately 500–700 ppm/%Brix haze dextran (Eggleston, Monge, Montes, & Stewart, 2007c). It can also be seen in Fig. 3 that there was a strong, polynomial relationship between mannitol and haze dextran ($R^2 = 0.912$). A polynomial fit was slightly better than a linear fit ($R^2 = 0.909$). In Louisiana, processing problems are predicted when ~ 1000 ppm/%Brix haze dextran occurs in crusher juice (Adrian Monge, Cora Texas Inc., personal communication), although this “threshold” value depends on the factory personnel. This corresponds to ~ 2500 ppm/%Brix mannitol (Fig. 3). However, this value can only be considered an *approximate predictor* of dextran because mannitol is produced by various *Lactobacillus* species and strains, although *Leuconostoc mesenteroides* is the greatest *Lactobacillus* producer of mannitol (Eggleston et al., 2007b). Consequently, mannitol can be considered a very sensitive measure of *Leuconostoc* and other *Lactobacillus* deterioration of sugarcane and a useful approximate measure of juice dextran (low, medium and high MW).

3.2. Relationship among mannitol, antibody dextran, and haze dextran in press and crusher juices

Although some factories prefer to measure antibody dextran using a commercial kit because it is more specific for dextran than haze dextran, the latter still remains more popular primarily because it is considerably less expensive to measure. Antibody dextran was measured on randomly selected press and crusher juices and compared to mannitol and haze dextran results (Figs. 4a and b). Consistently, much lower ppm/%Brix values were observed for antibody dextran than both haze dextran and mannitol (Figs. 4a and b), with mannitol values the highest (Fig. 4a). Antibody dextran measures high molecular weight (HMW) dextran (D. Day, Audubon Sugar Institute, personal communication) which contributes mostly to viscosity problems in the boiling house operations, whereas haze dextran measures high (>1000 KDa) and MMW (~ 100 –1000 KDa) dextran, MMW starch (if amylase is added), indigenous cane polysaccharide, and other haze forming

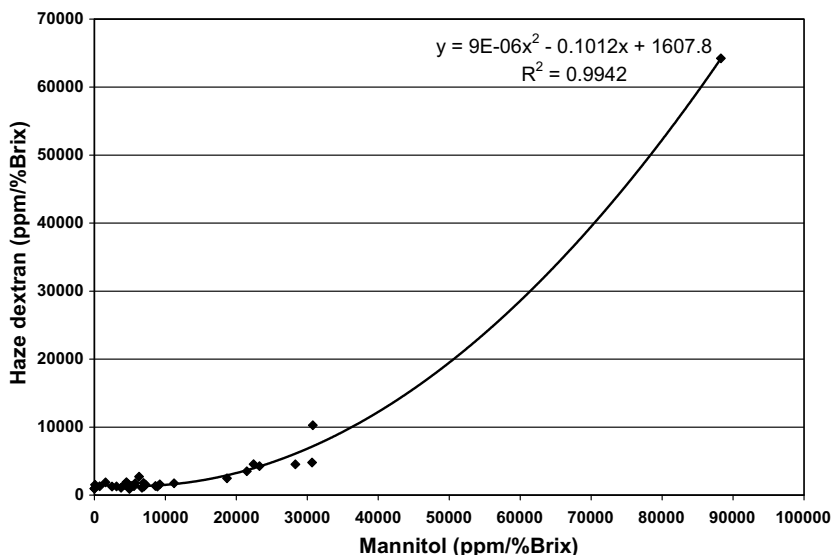


Fig. 4a. Relationship between haze dextran and mannitol in select press and crusher juices.

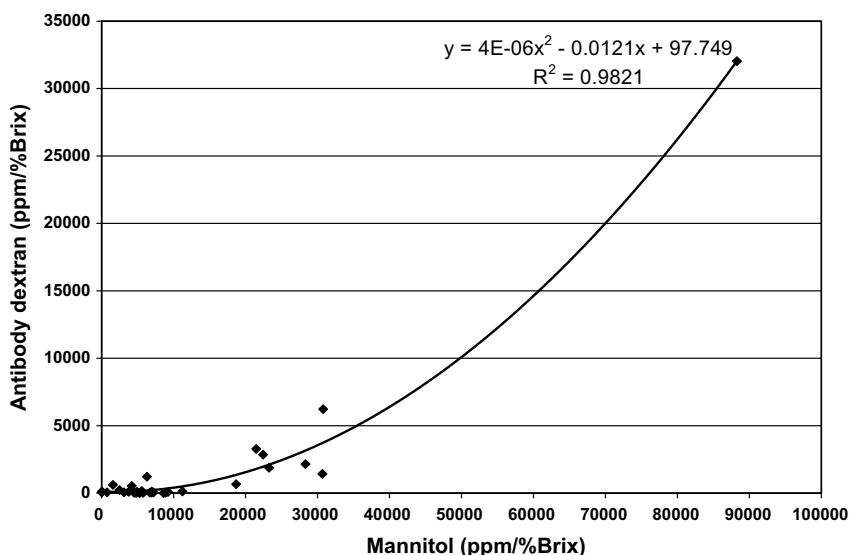


Fig. 4b. Relationship between antibody dextran and mannitol in select press and crusher juices.

compounds including microparticles (Eggleston et al., 2007c). Moreover, if other types of microbial deterioration have occurred then other microbial polysaccharides may also be present that can contribute to the haze.

From Fig. 4a, it can also be seen in select juices that a strong polynomial correlation ($R^2 = 0.994$) existed between mannitol and haze dextran, and between mannitol and antibody dextran ($R^2 = 0.982$), for both press and crusher juices (Fig. 4b). Again, polynomial fits (Figs. 4a and b) were better than linear fits indicating that a strong relationship exists between the increasing rate of change of mannitol and dextran formation, even though less dextran is formed than mannitol (haze dextran was higher than anti-

body dextran). As expected, the relationship between haze and antibody dextran was linear ($R^2 = 0.982$; Fig. 4c) because they are both a measure of the same compound. In comparison, mannitol is formed from a different metabolic pathway and enzyme in *Leuconostoc* than dextran (Eggleston et al., 2007b).

Like for the combination of both press and crusher juices (Figs. 3 and 4a) a strong polynomial relationship ($y = 2E-06x^2 + 0.0679x + 1039$; $R^2 = 0.923$) existed between haze dextran and mannitol in press juices only (Fig. 5a). In comparison, for crusher juices only, a more linear relationship ($y = 0.1759x + 261.4$; $R^2 = 0.928$) was observed. Presently, the authors have no explanation for this difference.

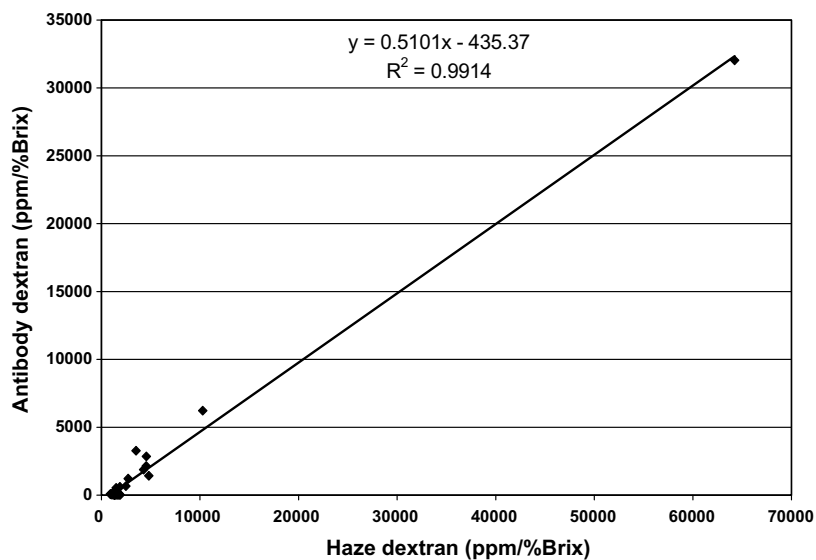


Fig. 4c. Relationship between antibody and haze dextran in select press and crusher juices.

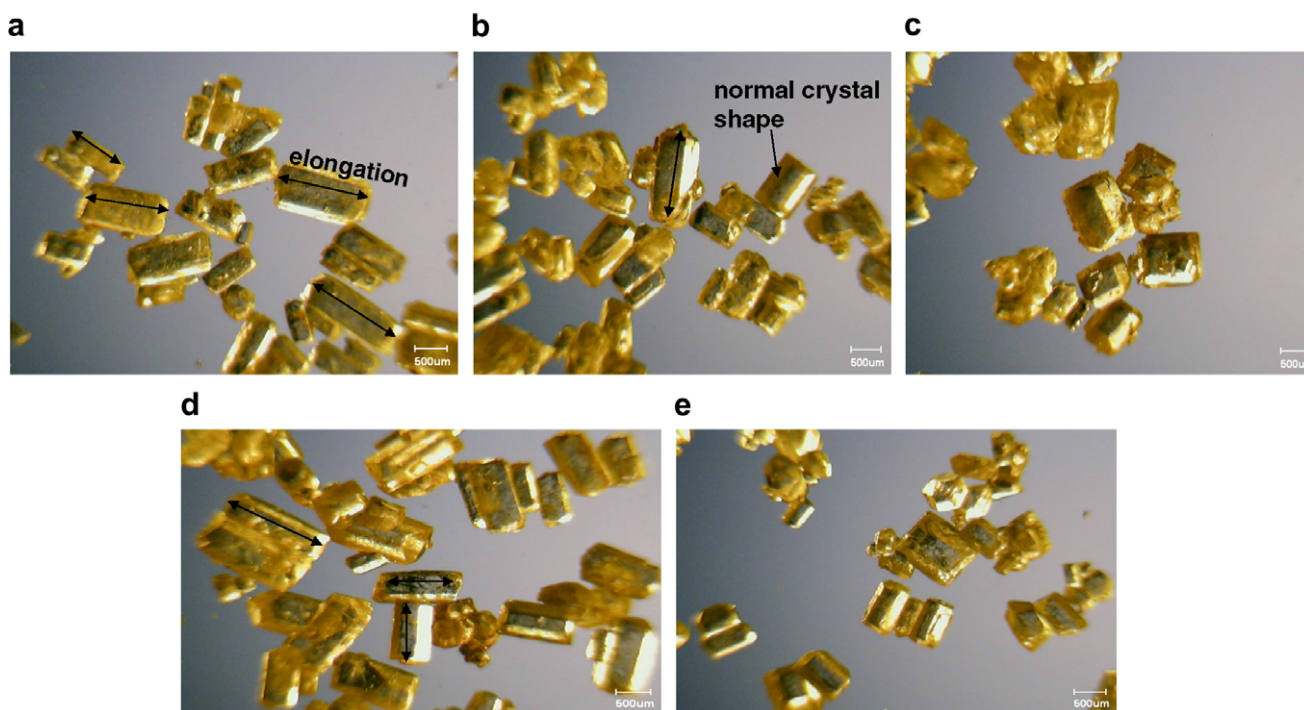


Fig. 5. Digital micrographs of raw sugars manufactured at the factory, (a) 21 October, 2004; crystal elongation, (b) 30 October, 2004; mixed crystal sizes, (c) 16 November, 2004; no crystal elongation, (d) 23 November, 2004; elongation, and (e) 2 December, 2004; no elongation.

4. Raw sugars

Digital micrographs of randomly selected raw sugars manufactured in the factory (Fig. 5) shows large variations in the crystal shape across the processing season, which indicated variations in the quality of the sugarcane that was processed. The occurrence of dextran in raw sugar crystals not only can cause the deformation of crystal shapes (Abdel-Rahman, Schick, & Kurz, 2007) but also cause the factory to be penalized by refineries. In the presence of dextran, sucrose crystals are often elongated across the *c*-axis (“needle grain”) because it influences the growth rates of individual crystal faces (see Fig. 5a, b and d). In particular, low molecular weight (LMW) dextrans exert more effect on crystal shape than HMW dextrans (Singleton, 2002). In comparison, HMW dextran detrimentally affects the growth rate of sucrose crystals more than LMW dextrans mostly because of its greater contribution to increased viscosity. The crystal shapes from raw sugar manufactured on 21 October (Fig. 5a) were elongated, which most likely reflects the relatively high temperatures experienced in the area before this date (Fig. 1); furthermore, the humidity was higher in October than December. On 30 October (Fig. 5b), mixed crystal shapes were observed, indicating not all the sugarcane being processed at the factory had been deteriorated. Although temperatures were still high up to 30 October (Fig. 1), the humidity was lower than earlier in the same month. On 16 November (Fig. 5c), normal crystal

shapes were observed in the raw sugar, which most likely reflects the lower temperatures in November and the very little rain in the area from 2 and 17 November (Fig. 1). However, by 23 November (Fig. 5d) elongated sucrose crystals had returned in the raw sugars, because of some previous days’ spikes in average temperature and rainfall (Fig. 1). On 2 December (Fig. 5e) no elongation was observed, which is most likely attributable to the marked lowering of the average temperature four days previously (Fig. 1).

A strong linear correlation ($y = 0.7278x + 183.48$; $R^2 = 0.832$) was calculated between haze and antibody dextran measurements in the raw sugars, and haze dextran was ~ 183 ppm/%Brix greater than antibody dextran. In a previous study by Saska, Godshall, and Day (2002) on 20 raw sugars, a similar correlation of $R^2 = 0.872$ was observed between haze and antibody dextran, with haze ~ 34 ppm/%Brix greater than antibody dextran. Preliminary measurements of mannitol using the enzymatic method on the raw sugars in this study often indicated there was no mannitol in the raw sugars, yet ion chromatography (IC) results indicated small amounts were present. Precision of the enzymatic mannitol method is known to be lower at small values of mannitol in both sugarcane (Eggleston & Harper, 2006) and sugar beet juices (Eggleston, unpublished results). Modifying the enzymatic method to have a longer delta absorbance allowed for increased detection of mannitol in the raw sugars, but the values obtained still did not correspond to the IC values. Therefore, more research is necessary

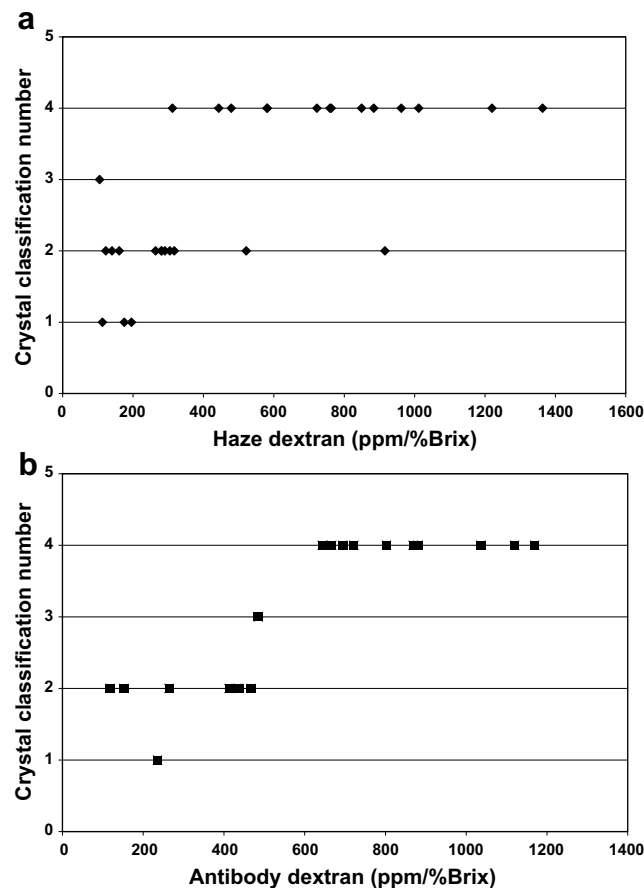


Fig. 6. Effect of haze dextran concentrations in raw sugars on subjective classification levels of crystal shape. (a) (1) Normal shaped crystals, (2) a mix of normal and slightly elongated shaped crystals, (3) a mix of normal and medium elongated shaped crystals, and (4) the majority of crystals are elongated. (b) Effect of antibody dextran concentrations in raw sugars on subjective classification levels of crystal shape.

to investigate the application of the enzymatic method in this different matrix. Problems with accuracy may be because of the formation of inhibitory compounds across factory processing that interfere with the enzymatic assay.

The raw sugars investigated in this study were classified roughly into four subjective categories: (1) normal shaped crystals, (2) a mix of normal and slightly elongated shaped crystals, (3) a mix of normal and medium shaped elongated crystals, and (4) the majority of crystals were elongated. These classification numbers were then plotted against haze dextran (Fig. 6a) or antibody dextran (Fig. 6b). Population clusters for each shape classification were more segregated and distinct with antibody than haze dextran (Fig. 6). Approximately >300 ppm/%Brix haze dextran in raw sugars started to indicate that the majority of the crystals being elongated (Fig. 6a). In comparison, >600 ppm/%Brix antibody dextran approximately indicated when elongated crystals were predominant in the raw sugar (Fig. 6b). The lower threshold value for haze dextran may be because it also detects low MW dextrans that are most responsible for crystal elongation.

5. Conclusions

Mannitol concentrations were easily measured in press (consignment) and crusher juices, from a sugarcane factory that processed different levels of deteriorated sugarcane across the processing season, using a recently developed, precise, accurate, rapid, and inexpensive enzymatic method (Eggleston & Harper, 2006). A strong, polynomial relationship existed between mannitol and haze dextran ($R^2 = 0.912$), and mannitol concentrations were consistently greater than haze and antibody dextran concentrations. In Louisiana sugarcane processing problems are predicted when ~1000 ppm/%Brix haze dextran occurs in crusher juice and this corresponds to ~2500 ppm/%Brix mannitol. Mannitol can, therefore, be considered the most sensitive measure of *Leuconostoc* and other *Lactobacillus* deterioration of sugarcane, an indicator of process problems, and a useful indirect and approximate predictor of juice dextran.

The enzymatic mannitol method is quantitative and results warrant further investigation of its use in sugarcane payment formulas that incorporate analysis results of different parameters in press juice. The inclusion of mannitol as a deterioration factor in the formula would serve as a deterrent to farmers in the delivery of excessively deteriorated sugarcane, as well as improve sugarcane management practices. Cost per analysis of the enzymatic mannitol method is much lower than the cost for rapid dextran analysis based on monoclonal antibody technology (Rauh et al., 2001), and mannitol has the further advantage over dextran of being an indicator of sugarcane deterioration because it can also indicate dextran, levan and other polysaccharides formed by *Leuconostoc* (Eggleston et al., 2004) and other bacterial contaminants. Mannitol was recently shown as a much more sensitive indicator of sugarcane freeze deterioration than (Legendre et al., 2007) dextran as measured by a tedious, enzymatic method (Sarkar & Day, 1986) and other deterioration parameters such as titratable acidity.

Approximately >300 ppm/%Brix haze in raw sugars indicated that the majority of the crystals were elongated. Approximately >600 ppm/%Brix antibody dextran indicated when elongated crys-

tals were predominant in the raw sugar. The enzymatic mannitol method underestimates mannitol in raw sugars. Until further research is undertaken on the effect of mannitol on industrial sucrose crystallization, dextran remains the analysis component of choice for raw sugars manufactured from deteriorated sugarcane.

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