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Determination of sugarcane deterioration at the factory: Development of a rapid, easy and inexpensive enzymatic method to measure mannitol

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Abstract

Mannitol, formed mainly by *Leuconostoc mesenteroides* bacteria, is a very sensitive indicator of sugarcane deterioration that directly affects processing and can predict problems from dextran and levan polysaccharides. An enzymatic method has been developed to measure mannitol in juice pressed from consignments of sugarcane delivered to the factory. This screening tool will allow factory staff to rapidly know which consignments of cane will affect processing negatively or reject consignments that will cause unacceptable processing problems. Mannitol is directly measured on a spectrophotometer using mannitol dehydrogenase as the enzyme catalyst. The stability of the reagents, limited cane juice preparation and linearity are described. This method is accurate, comparing favorably with an ion chromatography method, and can be easily performed using existing equipment in sugarcane factories. The coefficient of variation (CV) for cane juices ranged from 1.73% to 5.13% with the highest CVs occurring for low mannitol concentrations in undeteriorated cane. Mannitol can be measured after ~7 min at room temperature and within 4 min if a 40 °C waterbath is used. The method is highly specific for mannitol and was not affected by the presence of sucrose, glucose, fructose, or dextran. The current cost is only ~60 US cents per analysis. Further studies on the viability of the method at the factory, and as a screening tool for breeding programs for cane freeze tolerance, are being undertaken.
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Keywords: Mannitol enzymatic method; Mannitol dehydrogenase; Sugarcane deterioration

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 reliable, rapid, easy and inexpensive method to measure cane deterioration at the factory. This has meant that factory staff have not been able to screen individual consignments of cane and, thus, they do not know which consignments will detrimentally affect processing and are unable to reject unsuitable consignments of cane.

The major contributor to cane deterioration in the United States, particularly Louisiana where humid conditions prevail, is *Leuconostoc mesenteroides* lactic acid bacterial infections. *L. mesenteroides* bacteria are mostly known for producing dextran, a high viscosity glucopolysaccharide, that can reduce evaporation and crystallization rates. Current methods to determine dextran suffer from being either too long and complicated, not specific enough, too expensive (Rauh, Cuddihy, & Falgout, 2001), or there are difficulties in the interpretation of results (Clarke, Bergeron, & Cole, 1987).

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Numerous metabolic products other than dextran are formed by *L. mesenteroides* which are of importance in sugar manufacture, including levan (a fructose polysaccharide), alternan (a glucose polysaccharide), mannitol, leucrose, D- and L-lactic acids, and a series of isomaltooligosaccharides (Eggleston, Legendre, & Tew, 2004). Levan has been reported to cause viscosity problems at certain sugarcane and sugarbeet factories (Imrie & Tilbury, 1972). Mannitol, a sugar alchohol, does not degrade under processing conditions (Eggleston et al., 2004) and factory syrups and massecuites processed from deteriorated cane have been found to contain large amounts of it (Eggleston, Harper, & Kart, 2006). Furthermore, mannitol directly affects processing by reducing sugar recovery (Bliss, 1975) and evaporation rates (Eggleston et al., 2006). Mannitol has been repeatedly proven to be a sensitive measure of sugarcane deterioration (Eggleston, 2002; Eggleston & Legendre, 2002; Eggleston et al., 2004) and sugarbeet deterioration (Thielecke, 2002; Steinmetz, Buczys, & Bucholz, 1998). Eggleston et al. (2004) observed that mannitol predicted juice viscosity and pol filterability slightly better than dextran because it can indicate all *L. mesenteroides* polysaccharides, including dextran, levan, and alternan, and deterioration from mannitol forming *Lactobacillus* (Basso, 2005), although the latter is expected to be negligible. Overall, mannitol was the best predictor of cane deterioration which contributes to sucrose losses, dextran-related problems, viscosity problems and, to a lesser extent, filterability problems (Eggleston et al., 2004).

Previously in the sugar industry (Steinmetz et al., 1998; Eggleston et al., 2004), mannitol has been measured using the sophisticated technique of ion chromatography with integrated pulsed amperometric detection (IC-IPAD). IC-IPAD is not a possibility for use at the factory because of its expense, complication, and the level of expertise required of the operator. To be of any industrial use as a deterioration screening method, it is necessary that mannitol be easily, rapidly and inexpensively measured in juice pressed from individual consignments of cane. Consequently, it was decided to develop an enzyme method to measure mannitol at the factory. An enzymatic assay was chosen for its high specificity and because a chemical method would be too complicated, require unsafe chemicals and temperatures, and be too long (Corcoran & Page, 1947). Mannitol is currently determined clinically as an indicator of intestinal permeability, with mannitol being measured enzymatically in urine (Hessels et al., 2003; Lunn, Northrop, & Northrop, 1989), which is a more simple sample matrix than cane juice. Mannitol dehydrogenase (MDH) converts mannitol to fructose in the presence of the co-enzyme NAD^+, which acts as an electron acceptor:

$$\text{Mannitol} + NAD^+ \xrightarrow{\text{Mannitol dehydrogenase}} \text{Fructose} + NADH + H^+$$

The reduced form of the coenzyme, NADH, can be easily measured spectrophotometrically because it absorbs light in the ultraviolet region at 340 nm.

In this paper we describe the development of a rapid, enzymatic method using MDH that can be undertaken at the factory to measure mannitol in pressed cane juices, which represent individual sugarcane consignments. The stability of the reagents, linearity, precision, accuracy, and interference from other sugarcane sugars are reported.

2. Materials and methods

2.1. Chemicals, enzymes and juice samples

Mannitol dehydrogenase (EC 1.1.1.67) was purchased as a freeze-dried powder (8.45 U/mg dry weight) from Biocatalyst Ltd., Wales. All chemicals used were analytical grade. Mannitol, glycine, dithiothrietol, nicotinamide adenine dinucleotide (NAD), glucose, anhydrous glycerol, and potato starch were from Sigma (US), potassium dihydrogen phosphate (KH_2PO_4), fructose, and sucrose were from Baker (US), sodium hydroxide (NaOH) from Fisher (US) and dextran T2000™ (MW $\geqslant$ 2,000,000 Da) from G.E. Healthcare (US). A sugarcane juice with a very high dextran content was obtained for this study by allowing a sugarcane pile to deteriorate at ambient conditions for 3 days outside a Louisiana factory; juice was extracted in the factory laboratory core press. Three cane crusher juices were obtained from three other Louisiana factories. All juices (120 ml) were stored with 5 drops of biocide (Bussan 881™, Buckman Labs.), in a −60 °C freezer until used.

2.2. Buffers

To prepare glycine buffer (100 mM; pH 10.5), glycine (7.51 g) was dissolved in de-ionized water (800 ml) and adjusted to pH 10.5 with NaOH (1 M), then made up to 1 l with de-ionized water.

Phosphate buffer (25 mM; pH 6.0) with 30% glycerol was prepared by adding potassium dihydrogen phosphate (0.34 g) and glycerol (30 g) into a 100 ml volumetric flask, then dissolved in de-ionized water (50 ml) and adjusted to pH 6.0 with NaOH (1 M). Dithiothreital (15.4 mg) was added and the final volume made to 100 ml with de-ionized water.

2.3. NAD solution

NAD (0.22 g) was dissolved in 10 ml of de-ionized water; NAD solution has to be prepared daily.

2.4. Preparation of enzyme

A stock solution of enzyme was first prepared by dissolving 0.01 g of the freeze-dried MDH in 1 ml of ice cold phosphate + 30% glycerol buffer. For the assays, a further dilution was made by pipetting 100 μl of stock into a 10 ml volumetric flask and making to the final volume with phosphate + 30% glycerol buffer (10,000-fold dilution). Both the stock solution and diluted enzyme solutions were stored in a −20 °C freezer. The stock solution can be stored for ~1 month, and the diluted enzyme for 1–2 weeks. During analysis, the diluted enzyme was kept on ice at all times.

2.5. Mannitol dehydrogenase activity

Mannitol dehydrogenase (MDH) activity was based on the Biocatalyst method (Anon, 2003) with major modifications. One unit of MDH activity was defined as the amount of enzyme which catalyzes the reduction of 1 μmole of NAD^+ per min at 23 °C and pH 10.5. To two test-tubes glycine buffer (1.4 ml), mannitol (500 mM; 0.2 ml), and NAD (0.12 g dissolved in 5 ml of de-ionized water; 0.2 ml) were added. For the blank, 0.2 ml water added and the mixture immediately stirred on a vortex mixer, added to a 1 cm cuvette and placed in a spectrophotometer (Shimadzu UV-1201). For the test sample, 0.2 ml of 10,000X diluted MDH was added, vortex stirred then immediately added to a cuvette and the absorbance measured at 340 nm after 0, 1, 2, 3, 4 and 5 min. The increase in absorbance per min between 2 and 3 min was used for the activity calculation, based on Beer's Law:

$$U/\mathrm{ml} = \frac{A \times V_t \times d}{V_s \times E},$$

where A is Δ340 nm/min, V_t is the total volume of assay mixture (i.e., 2 ml), d the dilution of enzyme from original concentration (i.e., 100), V_s is the sample volume in assay mixture (i.e., 0.2 ml), E is the equimolar extinction coefficient of NADH (i.e., 6.22/ml of assay). Therefore: $U/\mathrm{mL} = \frac{4\times10\times100}{E} = A \times 160.8$. Therefore: $U/\mathrm{mg} = (\mathrm{A} \times 160.8)/C$, where C is the original enzyme concentration in mg/ml (i.e., 10).

2.6. Factory mannitol enzymatic method

The method was first standardized using five mannitol standards (1, 10, 100, 500 and 1000 ppm) diluted in de-ionized water, to generate a linear standard curve and equation. A new standard curve must be generated for each batch of enzyme. To measure mannitol in cane juice, the juice was first diluted 1:1 (i.e., 2-fold) in glycine buffer and then filtered through 0.45 μm pore-size PVDF (polyvinyldene fluoride) filter then a 0.22 μm pore-size PVDF filter. For difficult to filter samples, celite can be first added to the juice before filtering through the PVDF filters or the juice can first be filtered through Whatman™ 91 filter paper (185 mm; 10 μm). If 0.45 and 0.22 μm pore-size PVDF filters are not available then the juice can be prepared by adding 0.5 g of celite to 10 ml of cane juice in a syringe body, mixing well, filtering the juice through a glass filter (25 mm diameter), and discarding the first 2 ml of filtrate. The filtrate (5 ml) is then diluted with glycine buffer (5 ml).

To two test-tubes glycine buffer (1.4 ml), diluted and filtered juice (0.2 ml), and NAD (0.2 ml) were added. For the blank, 0.2 ml de-ionized water was added and the mixture, vortex stirred then immediately added to a 1 cm quartz cuvette and placed in a Shimadzu UV-1201™ spectrophotometer. For the test sample, 0.2 ml of 10,000-fold diluted MDH was added then immediately stirred on a vortex mixer then the timer started immediately. The solution was then added to a separate 1 cm cuvette, and the absorbance measured at 340 nm after 5 min. (Measuring the change in absorbance from 0 to 5 min is recommended). The final absorbance was the sample absorbance – blank absorbance. Calculations were based on the equation of the standard curve and dilution factors. For deteriorated juices containing high amounts of mannitol which cause the mannitol absorbance to be higher than the upper limit of the standard curve, further dilutions of 1:3 (4-fold) or 1:7 (8-fold) in glycine buffer are required.

Sample test-tube	Blank test-tube
1.4 ml glycine buffer	1.4 ml glycine buffer
0.2 ml diluted and filtered juice	0.2 ml diluted and filtered juice
0.2 ml NAD	0.2 ml NAD
0.2 ml MDH enzyme	0.2 ml water

2.7. Effect of temperature on the factory enzymatic method

The factory mannitol method was followed except 0.2 ml of mannitol (1400 ppm) replaced the juice and, after the enzyme was added the two test-tubes were placed in a shaking (90 rpm) waterbath (Julaba SW22) at different temperatures (23–48 °C) for 5 min, before the absorbance was measured.

2.8. Effect of added sugars on the factory enzymatic method

Model solutions were made to simulate levels of sugars in a typical sugarcane juice, and all the solutions had a final °Brix of 14.0. All the solutions contained either 1000 or 2000 ppm mannitol. Glucose and fructose additions were on ~3% on solids basis, su-

crose ~90% solids. Dextran was added at 1000 ppm level. The factory mannitol method was followed except the model sugar containing mannitol (1000 or 2000 ppm) replaced the juice.

2.9. Haze dextran in sugarcane juices

Haze dextran in juices was based on the modified method of Eggleston and Monge (2005). Termamyl™ (Novo, US) amylase enzyme was added to remove interfering starch. Dextran T2000™ was the standard and dextran was precipitated with 100% absolute ethanol. The mean °Brix of triplicate samples was measured using an Index Instruments TCR 15–30 temperature controlled refractometer accurate to ±0.01 °Brix.

2.10. Mannitol determined by ion chromatography with integrated pulsed amperometric detection

See Eggleston (2002) for method. Dilutions varied, depending on the juice, from 1 g/50 ml to 1 g/500 ml.

2.11. Statistics

Single factor ANOVA was conducted using Microsoft Excel™, version 2002 with SP-2.

3. Results and discussion

3.1. Stability of the enzyme

In our initial research we found problems with the stability of the enzyme which had been diluted in a phosphate buffer without glycerol, even if the stock or diluted enzyme was stored in a −40 °C freezer. This is illustrated in Fig. 1(a). After 8 days of storage in a −20 °C freezer the MDH activity had markedly decreased from 1.88 to 0.20 U/ml. We undertook stabilization studies and found the addition of glycerol to the buffer stabilized the enzyme stored as both stock or as a further diluted solution. There was a slightly higher level of stability of the enzyme diluted from stock that had been stored in buffer containing 30% glycerol, compared to that from stock that contained no glycerol, and this increased stability was observed even in fresh, un-stored preparations (day 1). As can be seen in Fig. 1(b), both the preparation of the enzyme stock, and diluted enzyme from the stock, need to be undertaken in phosphate buffer with glycerol. Glycerol at the 30% level was found to be optimum (Fig. 1(b)). The freeze-dried enzyme is stable in a −20 °C freezer for up to 6 months. The enzyme stock and diluted enzyme can be stored in conventional −20 °C freezers.

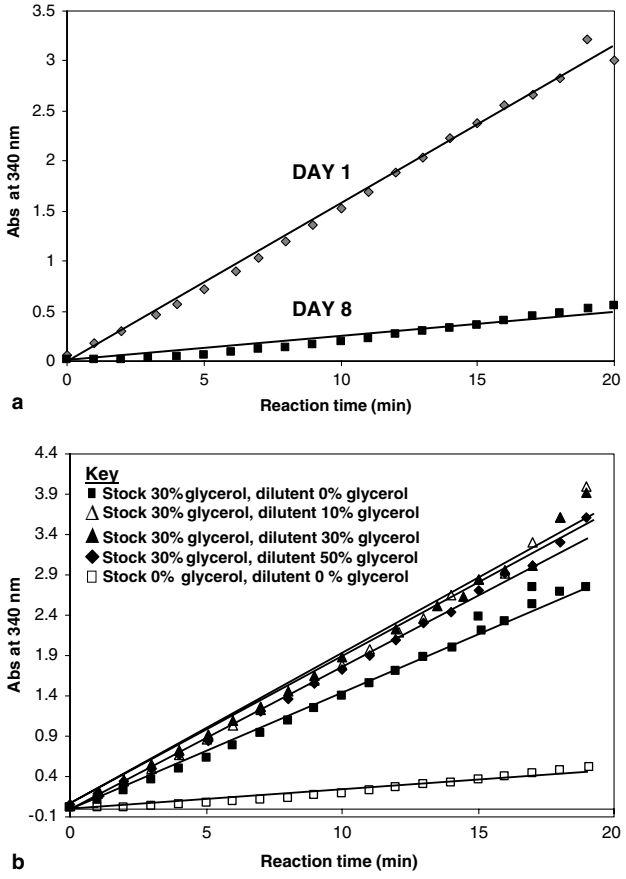

Fig. 1. (a) Instability of MDH when the stock and diluted enzyme solutions were prepared in phosphate buffer only with no added glycerol. (b) Stabilization of MDH after 8 days when the stock and diluted enzyme solutions were prepared in phosphate buffer, with added glycerol at different concentrations.

3.2. Interfering particles in cane juice

Cane juice is a complex matrix and contains numerous large particles, including bagacillo fibers, soil, and starch granules. We found that particles in undiluted and unfiltered cane juice can interfere greatly with the enzymatic mannitol determination. Cane juice must be diluted for this assay and usually a minimum 1:1 (2-fold) dilution in glycine buffer is adequate, unless the juice is highly deteriorated and then further dilutions are required. Filtering of the diluted juice is also necessary, and we observed that filtering through a 0.22 μm pore-size PVDF filter is better than through a 0.45 μm pore-size PVDF filter, which is illustrated in Fig. 2. The 0.45 μm pore-size filter was not able to remove enough interfering particles, causing not only interference but inhibition of the assay (Fig. 2).

3.3. Effect of temperature

In our early efforts to develop a simple method to determine mannitol in sugarcane consignments, we were

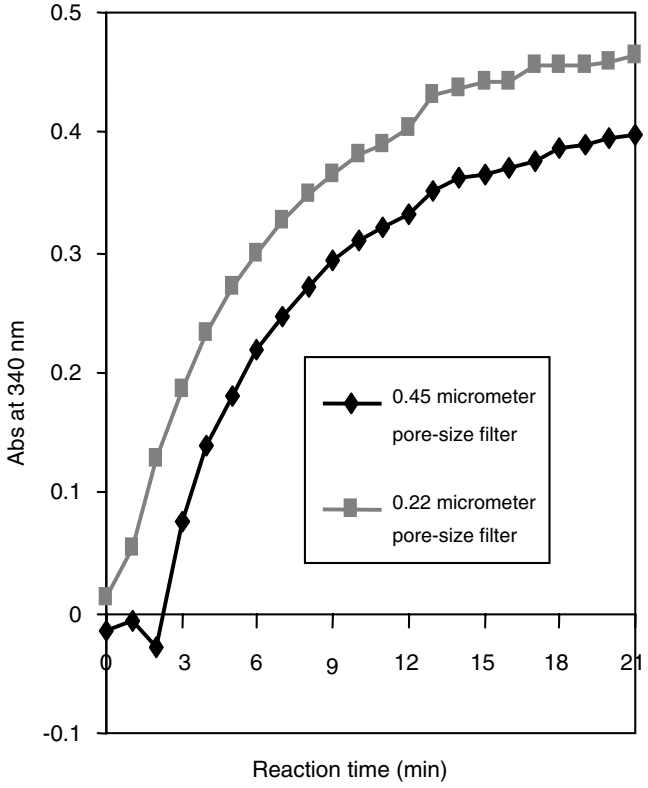

Fig. 2. Effect of filter pore size on removing interfering particles in the mannitol enzymatic assay (conditions: cane juice; 1:1 (2-fold) dilution).

concerned that the method was also relatively rapid. For this reason, we investigated the effect of temperature on MDH activity, which is shown in Fig. 3. However, although the activity of MDH is optimum between 37 and 43 °C (Fig. 3), incubating the enzyme/juice mixture in a waterbath at the factory would only add another level of complexity and cost. We, therefore, decided to keep the reaction at room temperature (23 °C) for the final factory method. Nevertheless, if factory staff want an even faster method, they can incubate the enzyme/juice mixture in a waterbath at 40 °C for 2 min, instead of leaving it at room temperature for 5 min.

3.4. Linearity

The relationship between the mannitol concentration and the absorbance at 340 nm after 5 min was found to be only approximately linear up to 1000 ppm, which may be due to lack of substrate at very lower levels, and product inhibition ~1000 ppm level. Therefore, up to the 1000 ppm level, a quadratic fit would be better. These results are in approximate agreement with Lunn et al. (1989) who used a different source of mannitol dehydrogenase. We found a better linear fit, from 1 to 500 ppm mannitol, which is shown in Fig. 4.

3.5. Precision of the enzymatic factory method to determine mannitol in cane juices

The method precision was very acceptable in four cane juices assayed (Table 1), with coefficients of variation ranging from 1.73% to 5.13%. The precision tended to become worse when relatively lower amounts of mannitol were present in slightly deteriorated and undeteriorated cane juices (Table 1).

3.6. Determination of mannitol in the presence of other cane sugars

Sugarcane juices have high levels of glucose, fructose, and particularly sucrose. Dextran will also often be present when mannitol is present in cane juice because both are formed mainly from *L. mesenteroides* (Eggleston, 2002). These short and long chain carbohydrate sugars could potentially interfere with the measurement of

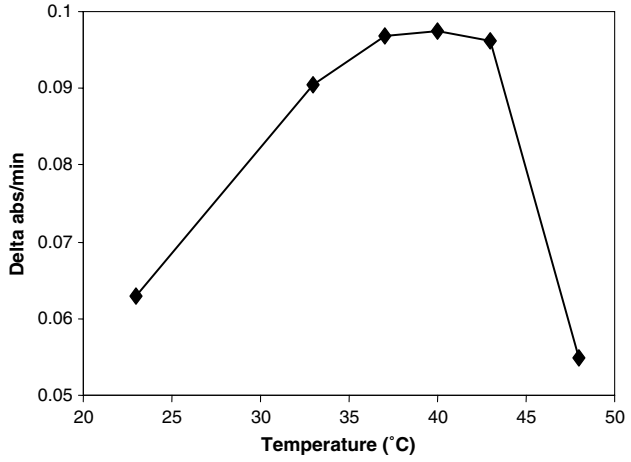

Fig. 3. Effect of temperature on the activity of mannitol dehydrogenase.

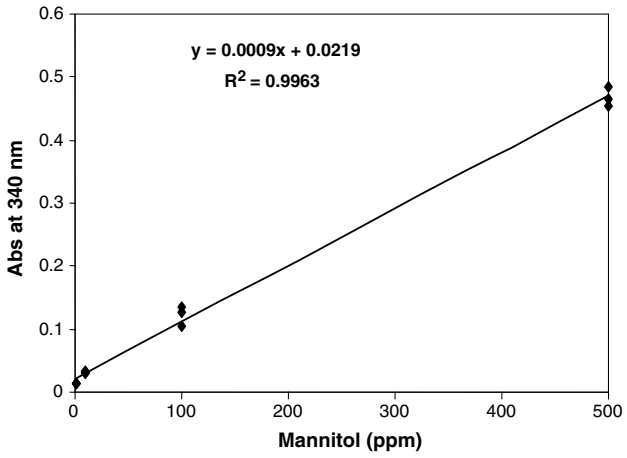

Fig. 4. The linear relationship between mannitol concentration and absorbance at 340 nm after 5 min incubation at room temperature.

Table 1
Precision of the enzymatic method for the determination of mannitol in sugarcane pressed juices expressed as the coefficient of variance (CV)

Cane juice sample	Juice °Brix	*N*	Mean concentration of mannitol (ppm)	Mannitol variation CV (%)	Haze dextran (ppm)
Deteriorated, pressed cane juice[a]	14.89	10	20,455	3.32	4688
Crusher juice from factory A	14.70	8	3870	4.50	585
Crusher juice from factory B	16.01	10	3259	5.32	204
Crusher juice from factory C	14.67	9	7180	1.73	944

[a] Had to be diluted 8-fold.

Table 2
Determination of mannitol in the presence of other sugarcane sugars in simulated 14.0 °Brix juices

Simulated juice[a]	Mannitol conc. added (ppm)	Mean % recovery of mannitol[b,c]	Total variation CV (%)[c]
Sucrose	2000	100.6	1.86
Sucrose + glucose	2000	102.5	3.16
Sucrose + glucose + fructose	2000	99.6	3.28
Sucrose	1000	103.6	2.97
Sucrose + glucose	1000	104.2	3.26
Sucrose + fructose	1000	103.6	1.36
Sucrose + glucose + fructose	1000	99.1	7.16
Sucrose + glucose + fructose + dextran (diluted 1:2; 7.0 °Brix)	1000	100.5	1.63

[a] See experimental section.
[b] $N = 3$.
[c] No statistical differences were found amongst the simulated juices with different sugars added using single factor ANOVA.

mannitol. We, therefore, investigated the effect of different sugars as they approximately occur in sugarcane juices, using simulated, model juices and results are shown in Table 2. None of the sugars showed any statistical interference at the 95% probability level with the mannitol determination, which confirms the ability of the method to accurately measure mannitol in cane juices. The analytical recoveries were very acceptable between 99.1% and 104.2% (Table 2).

3.7. Accuracy

The accuracy of the developed enzymatic method to measure mannitol in cane juices was determined by comparing the results with an ion chromatography (IC-IPAD) method (Fig. 5). An excellent correlation existed ($R^2 = 0.99$) between the two methods, that validates the accuracy of the enzymatic method. Furthermore, there were no significant differences at the 95% probability level, between the two methods for any of the juices analyzed. Precision was, however, worse for both methods in the juice containing <550 ppm mannitol (Fig. 5), which reflects the difficulty of detecting small amounts of mannitol, and confirms results listed in Table 1. As factory staff are more concerned with detecting mannitol concentrations >550 ppm in deteriorated pressed cane juice at the factory, this lower precision should have limited impact at the factory.

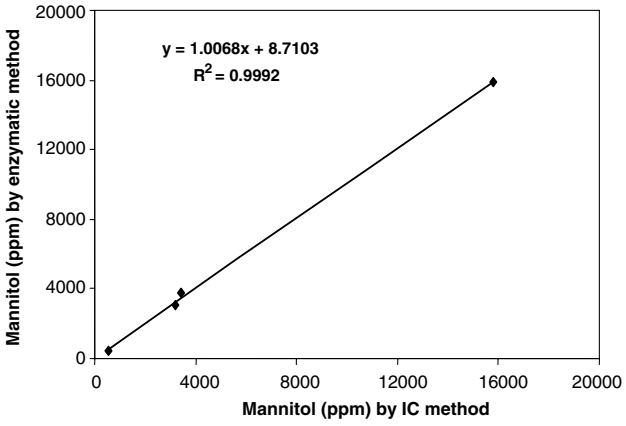

Fig. 5. Linear correlation between the enzymatic method for determining mannitol and cane juices and an ion chromatography (IC) method. No statistical differences between the two methods were found at the 95% probability level for any cane juice studied.

3.8. Relationship of mannitol with dextran measured by the haze method

As well as mannitol concentrations, Haze dextran concentrations in four cane juices are shown in Table 1, and the correlation between Haze dextran and mannitol was excellent at $R^2 = 0.99$ at the 99% probability level. This confirms previous observations from a laboratory cane deterioration study (Eggleston, 2002)

where mannitol, as measured by IC, correlated similarly ($R^2 = 0.98$) with dextran that was measured by an accurate enzymatic method. In more complex field studies of cane deterioration (Eggleston & Legendre, 2002; Eggleston et al., 2004), other strong correlations were found between mannitol measured by IC or enzymatically and Haze dextran, but with slightly lower R^2 values of 0.84 which reflects the further complexity of the multiple field samples representing numerous cane varieties.

As can be seen in Table 1, mannitol concentrations were markedly higher than Haze dextran concentrations. Higher mannitol than dextran concentrations have been previously observed in deteriorated juices from different cane varieties (Eggleston & Legendre, 2002; Eggleston et al., 2004) and in juices from *Rhizoctonia* affected beets that were susceptible to *L. mesenteroides* deterioration in the late stages (Bruhns, Lemmes, & Schick, 2004). This not only highlights the usefulness and higher sensitivity of mannitol to better predict *L. mesenteroides* deterioration than dextran, but the underestimation by sugar industry personnel of the relatively large amounts of mannitol present in deteriorated cane that will affect processing.

4. Conclusions

An enzymatic method has been developed to measure mannitol and, therefore, the extent of deterioration in juice pressed from consignments of sugarcane delivered to the factory. This enzymatic method has many advantages for the factory staff. These include ease and speed (~7 min at room temperature and within 4 min if a 40 °C waterbath is used to incubate the juice) of the determination. Time between assays can also be saved if disposable cuvettes are used. Also the method can be easily performed using existing equipment at the factory. The method is accurate and precise, and is highly specific for mannitol and was not affected by the presence of sucrose, glucose, fructose, or dextran. Furthermore, the current cost per analysis of mannitol in a sugarcane load at the factory is only ~60 US cents. The largest cost is the NAD at 45 cents per analysis. The MDH cost 12.5 cents per analysis. This cost per analysis is markedly 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 cane deterioration because it can also indicate dextran, levan and other polysaccharides formed by *L. mesenteroides* (Eggleston et al., 2004), as well as deterioration from mannitol forming *Lactobacillus* (Basso, 2005) although the latter is expected to be negligible. This enzymatic method also offers a valuable tool to cane breeders to screen varieties more rapidly, precisely and accurately in breeding programs for cold (freeze deterioration) tolerance in cane. Further studies on the viability of the method at the factory, and as a breeding tool are being undertaken.

Acknowledgements

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