

# HPAEC: SOME PRECAUTIONS REQUIRED FOR THE RELIABLE ANALYSIS OF CARBOHYDRATES

KJ SCHÄFFLER, PG MOREL DU BOIL AND SN WALFORD

*Sugar Milling Research Institute, University of Natal, King George V Avenue, Durban, 4001*

## Abstract

Although high performance anion exchange chromatography (HPAEC) is used routinely in several research and routine laboratories, results can sometimes be disappointing. Consistent retention times are necessary when using the peak height mode for quantitation, necessitating the use of a thermostatted oven. Detector response is also temperature dependent and, even with airconditioning, linearity can be poor. A thermostatted housing for the detector and an efficient airconditioned room have resulted in good detector linearities over restricted concentration ranges. The accurate measurement of low levels of invert in raw and white sugar was compromised by sucrose inversion while the sample vials were queuing for analysis. Addition of a biocide and sodium hydroxide resulted in greatly improved results. Analysis of sucrose in mixed juice indicated that invertase activity was occurring in the diluted samples (particularly from mills rather than from diffusers) prior to injection. Procedures for denaturing the samples are outlined.

**Keywords:** Sugars, HPLC, Carbohydrates, Electrochemical Detection

## Introduction

Anion exchange chromatography coupled with pulsed amperometric detection has been used for some time at the Sugar Milling Research Institute (SMRI) for measuring glucose, fructose and sucrose in molasses and other products. The procedure was recently adopted as an official analytical method by the International Commission for Uniform Methods of Sugar Analysis (ICUMSA) (Schäffler, 1994) and has been granted First Action Official Method status by the Association of Official Analytical Chemists (AOAC) (Anon, 1996). The method is relatively simple and is generally precise and accurate. The bracketing technique suggested by Thompson (1990) is used at the SMRI and lactose is added as an internal standard. Arabinose (Corradini *et al.*, 1993) or leucrose (Thielecke, 1994) have also been found to be suitable internal standards. A quality assurance programme similar to that introduced for the routine GC analysis of molasses (Schäffler and Day-Lewis, 1983), is in operation. However, an increased incidence of unacceptable results, together with less linear calibration curves during the 1994-95 season, prompted an in-depth investigation into the causes of these problems. Of particular concern was the possible effect on the introduction of HPAEC for mixed juice analysis. This paper describes some of the findings.

## Experimental

Chromatographic instrumentation included:

**Autosamplers:** SpectraPhysics SP 8875 with Rheodyne 7010 valve  
SpectraPhysics AS 3000 with Rheodyne 7010 valve  
SGE 3200LS with Valco AC6W valve  
Manual injection using Rheodyne 7125 valve

**Pumps:** Waters M-45  
SpectraPhysics IsoChrom  
SpectraPhysics SpectraSeries P100  
SpectraPhysics SpectraSystem P2000

**Columns:** CarboPac PA1 (Dionex) (250 mm x 4 mm)  
CarboPac PA1 Guard column (Dionex) (25 mm x 3 mm)

**Column heaters:** Column heater with controller (Omron ESC4) manufactured in SMRI workshop (controlled at 28°C)

**Detectors:** Dionex Model PAD - 2  
ESA Coulochem II

**Cells:**

- Dionex standard PAD cell with gold electrode and silver reference electrode (filled with sodium hydroxide) (Dionex STD-PAD-2)
- Dionex solvent-compatible PAD cell with gold working electrode and replaceable Ag/AgCl reference electrode (Dionex SC-PAD-2)
- ESA Model 5040 carbohydrate cell with gold working electrode and palladium reference electrode (ESA 5040)

**Detector settings:** The initial detector settings were based on the manufacturers' recommendations and are shown in Table 1. Subsequent adjustments are discussed later.

**Detector heaters:** Detector heater with controller (Omron ESC4) manufactured in SMRI workshop (controlled at 28°C).

**Integrators:** HP 3396A  
HP 3396 Series II  
EZChrom chromatography data system.

**Table 1**  
Manufacturers' recommended detector settings

Detector setting	Function	Cell		
		Dionex STD-PAD-2 (i)*	Dionex SC-PAD-2 (ii)*	ESA 5040 (iii)*
E <sub>1</sub> (mV)	Anodic	50	50	200
t <sub>1</sub> (ms)	detection:	300	480	500
AD (ms)**	Delay	(100)	(100)	300
SP (ms)**	Signal sampling	200	200	(200)
E <sub>2</sub> (mV)	Oxidative	650	600	700
t <sub>2</sub> (ms)	cleaning	60	120	100
E <sub>3</sub> (mV)	Reductive	-950	-600	-900
t <sub>3</sub> (ms)	reactivation:	180	60	100
Range (kNA)		10	30	

\* Reference: (i) Schäffler (1994); (ii) Schäffler and Day-Lewis (1992); (iii) Anon (1994); Bowers (1991)

\*\* AD = Acquisition delay; SP = Sampling period

The preparation of samples and calibration standards has been described elsewhere (Schäffler, 1994) and consisted of dilution and filtration through a 0,45 µm cellulose acetate (or similar) membrane. Amendments are discussed in this paper.

**Results and discussion**

The successful application of HPAEC techniques to quantitative analysis relies on attention to detail. The following discussion highlights some of the precautions and instrument fine-tuning which have contributed to the improved reliability of carbohydrate analysis when using HPAEC.

*(A): General*

The usual laboratory practices associated with high performance liquid chromatography (HPLC) need to be observed. Solvents must be prepared from high quality water, and carbon dioxide must be excluded from the highly alkaline mobile phase at all times. This is accomplished by continuous sparging with helium. Stock solutions of sodium hydroxide should be aged for at least five days before being used for solvent preparation to ensure complete precipitation of carbonate. The pellicular nature of anion exchange phases allows rapid solute-solvent equilibration, but limits column loading. However, as will be shown in later discussion, detector linearity has a more limiting influence on sample size than does column capacity. A single 250 mm column together with a guard column has been found to give adequate capacity for the relatively simple separations encountered in the sugar industry. Column fouling has occurred occasionally, but the system has been readily regenerated with 500 mM sodium hydroxide. Timeous regeneration or replacement of the guard column has protected the main column. The column currently in use for the routine analysis of molasses has had over 13 000 injections with no discernible deterioration in performance. The high purchase price of these columns is offset by their ruggedness and long life. Good temperature control has been found to be an important factor for continued reliable performance.

**Table 2**  
Effect of column temperature on resolution

NaOH conc (mM)	Column temperature (°C)	Resolution		N (sucrose)	Retention (sucrose) (min)
		Glucose/fructose	Lactose/sucrose		
150	24	1,56	2,68	4 750	8,4
	27	1,81	2,15	5 275	7,7
	30	1,87	1,73	4 525	7,4
	35	1,96	1,26	5 300	6,9
	38	2,25	0,66	4 115	6,5
125	30	2,23	1,00	4 635	8,0
	38	2,42	-	-	7,3

*Column temperature and resolution*

Consistent peak retention times are necessary for quantitation by means of peak height. Uniform pump flow rates and good column temperature control are thus essential. Although increasing the column temperature improves the glucose-fructose resolution, the lactose-sucrose resolution is adversely affected (Table 2). Hence, for good resolution of lactose (the internal standard) and sucrose the column temperature should be as low as can be adequately controlled. An

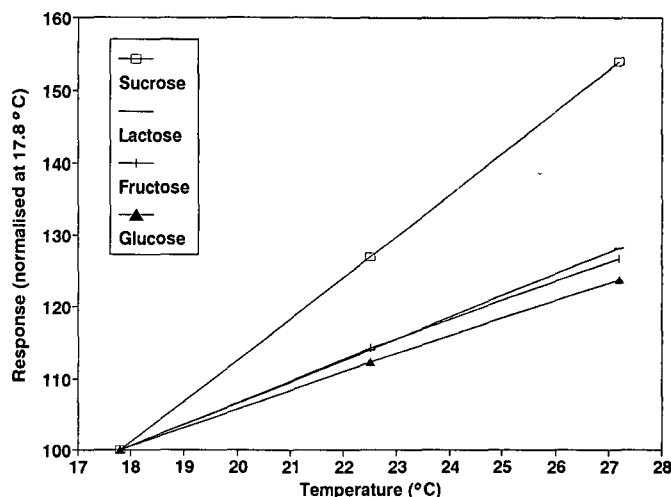
oven is used to maintain a constant column temperature slightly above ambient (27 to 28°C). The use of an internal standard (in this case lactose) does not adequately compensate for variable retention times since column temperature affects the relative retention (and hence relative peak heights) differently. Resolution can, to some extent, be influenced by solvent strength. Sodium hydroxide, in the range 100 to 150 mM, is usually used. At sodium hydroxide concentrations below 100 mM, problems with carbonate build-up on the column can be expected. It can also be seen in Table 2 that 150 mM sodium hydroxide gives better resolution of the lactose-sucrose pair than does 125 mM (at 30 to 40°C). Thompson (1990) found that 250 mM sodium hydroxide increased the selectivity between lactose and sucrose.

*(B): Detector behaviour*

Several factors can influence the sensitivity and reliability of amperometric detection. Cell geometry and design are dictated by the manufacturer and, to a large extent, one has to be guided by their recommendations for potential and pulse settings. The suitability of some of these conditions will depend on the required analytical constraints. The following factors have been found to influence detector behaviour.

*Temperature control*

The amperometric detector response is a rate-dependent electrochemical reaction. This rate will be dependent on electrode surface area, the distance between the working and reference electrodes, liquid flow rate, eluent concentration and cell temperature. Different reference materials and different sugars have different temperature coefficients. It can be seen in Figure 1 (using a Dionex STD-PAD-2 cell) that sucrose response was more affected by temperature than that of the other three sugars. Furthermore, Figures 2 and 3 show that both absolute response and relative response (to lactose) for fructose and sucrose changed in sympathy with the changing detector temperature. These results were obtained during an actual run in an airconditioned laboratory and so the temperature range was more limited. Such temperature fluctuations were subsequently avoided by placing the detector in a thermostatted oven.



**FIGURE 1:** Effect of temperature on detector response for sugars (using Dionex STD-PAD-2 cell)

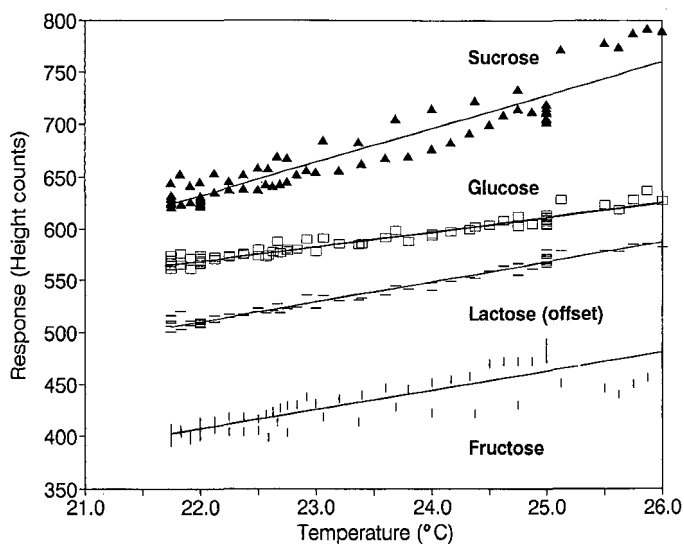


FIGURE 2: Effect of detector temperature on absolute response for sugars in final molasses calibration standard

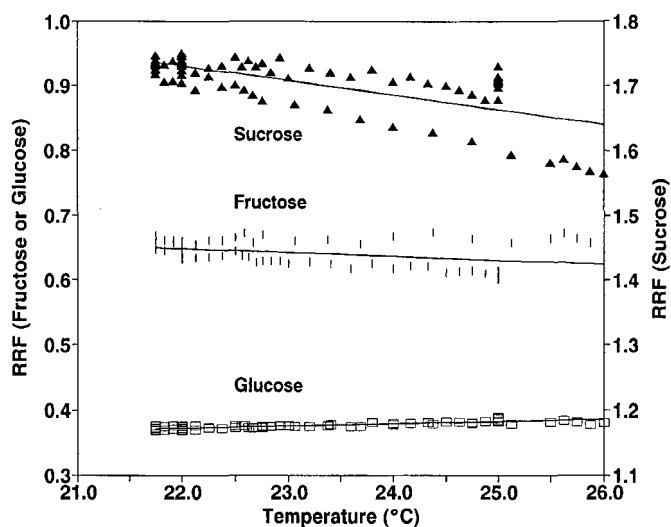


FIGURE 3: Effect of detector temperature on relative response factors for sugars in final molasses calibration standard

LaCourse and Johnson (1993) have observed that large temperature fluctuations can lead to significant changes in the sensitivity of pulsed amperometric detector (PAD) response – especially for compounds with oxidation rates under mixed or surface control, e.g. fructose (mixed) and sucrose (surface). Experience at the SMRI has confirmed that consistent sucrose response factors (and, to a lesser extent, fructose response factors) can be difficult to achieve if the detector is exposed to temperature fluctuations.

*Optimisation of potential settings*

Potential and pulsing programmes were initially those recommended by the manufacturers. Pulsed amperometric detection is controlled by a series of multi-step potential-time waveforms to maintain uniform and reproducibly high electrode activity for electrocatalytic oxidation. Because of the complexity of the pulse programme (seven interdependent parameters are involved) one has to be guided by the manufacturer’s recommendations when setting up the detector (Anon, 1989; 1993a; 1993b; 1994). However, some manufacturers have more expertise in carbohydrate applications than

others and some fine-tuning can lead to better control. It is not always clear from published data whether applied potentials are relative to the reference in use or to some other standard such as the standard calomel electrode (SCE). In addition, uncertainties associated with liquid junction and membrane diffusion potentials mean that corrections are not always straightforward. The reaction mechanisms and rate-controlling steps of the detection process have been studied in some detail (LaCourse and Johnson, 1991; 1993) and a useful review has been published (Johnson and LaCourse, 1990). Some guidelines have also been published by Andrews and King (1990). Adjustments to the initial detector settings are discussed in this paper.

When the ESA 5040 cell was used to measure sugars in mixed juice, two problems were encountered:

- additional PAD responsive components were noticed
- a significant (usually negative) baseline disturbance was observed. The elution volume of this peak was unaffected by solvent composition (e.g. inclusion of sodium acetate) or column temperature. The peak was enhanced if the sample was aerated and absent if the sample was sparged with either N<sub>2</sub> or He. Professor Johnson (<sup>1</sup>personal communication) speculates that this peak arises as a consequence of the pH fluctuation resulting from oxygen reduction at E<sub>3</sub> and suggests that increasing t<sub>1</sub> can nearly eliminate this ‘oxygen interference’.

The following effects were investigated at the SMRI:

- E<sub>1</sub> – the measuring (or detection) potential. It can be seen from Figure 4 that the peak eluting at about 3,5 minutes was not detected when the applied potential was decreased from 200 to 50 mV. While this particular peak is well resolved and does not interfere with the measurement of glucose, fructose or sucrose, it could well co-elute with one of the other monosaccharides. Similar compounds might co-elute with the sugars of interest. Clearly detector specificity towards carbohydrates can be improved by selecting a lower applied potential for E<sub>1</sub>. Data presented in Figure 5 show that sensitivities for glucose, fructose and lactose (in particular) were not greatly affected at lower

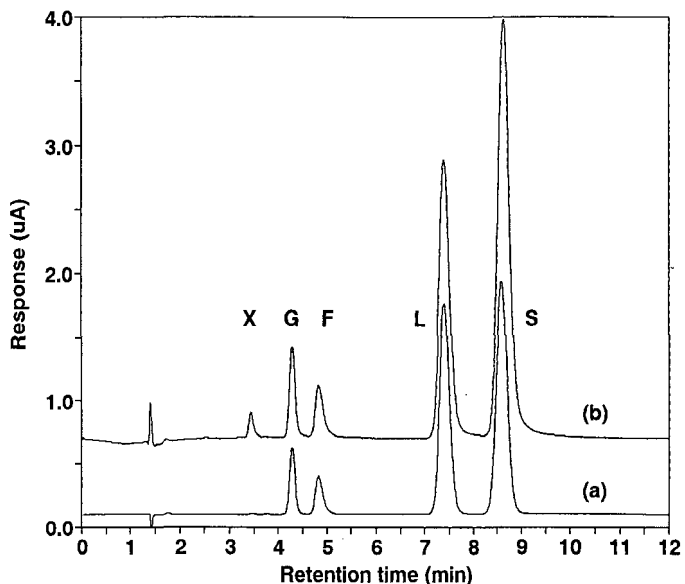


FIGURE 4: Specificity of PAD for carbohydrates (using ESA 5040 cell) with (a) E<sub>1</sub> = 50 mV (b) E<sub>1</sub> = 200 mV (X = unknown; G = glucose; F = fructose; L = lactose; S = sucrose)

<sup>1</sup>Prof Dennis C Johnson, Dept of Chemistry, Iowa State University, Ames, IA 50011, USA

settings for  $E_1$ . The background current was also lower. Although sucrose was affected to a greater extent, it is generally the major component and detection limits do not pose a problem. Interferences from juice components can be minimised by using lower values of  $E_1$ . A value of 50 mV was chosen for subsequent work.

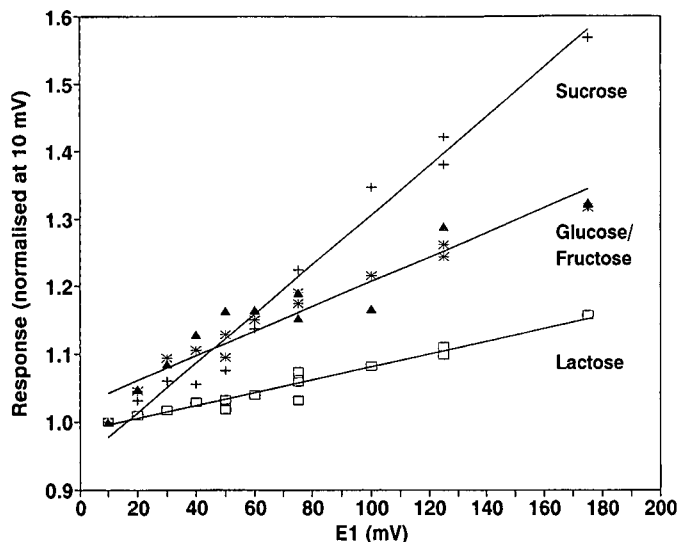


FIGURE 5: Effect of  $E_1$  on detector response for sugars in mixed juice using ESA 5040 cell.

•  $E_3$  and  $t_3$  – the conditioning (or reactivation) pulse. It was found that the ‘oxygen interference’ could be almost eliminated by increasing  $E_3$  values to -200 to -400 mV (Figure 6). Since it was felt that less severe conditions would decrease the reduction of oxygen,  $t_3$  was varied while holding  $E_3$  at -200 mV. It can be seen from Figure 7 that, as  $t_3$  increased, so did the peak interference. Hence a conditioning potential of -200 mV with a conditioning time of 300 ms was chosen. The interference can also be minimised by injecting smaller sample aliquots (i.e. less ‘dissolved oxygen’ available for reaction). This effect is shown in Figure 8.

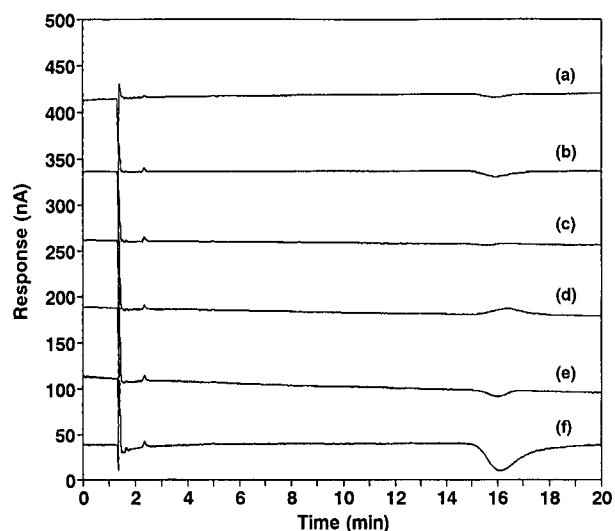


FIGURE 6: Effect of  $E_3$  on ‘oxygen disturbance’ at 16 minutes for a water injection ( $20 \mu\text{l}$ ) with  $t_3 = 150 \text{ ms}$  and  $E_3 =$  (a) -200 mV; (b) -300 mV; (c) -400 mV; (d) -500 mV; (e) -600 mV; (f) -800 mV

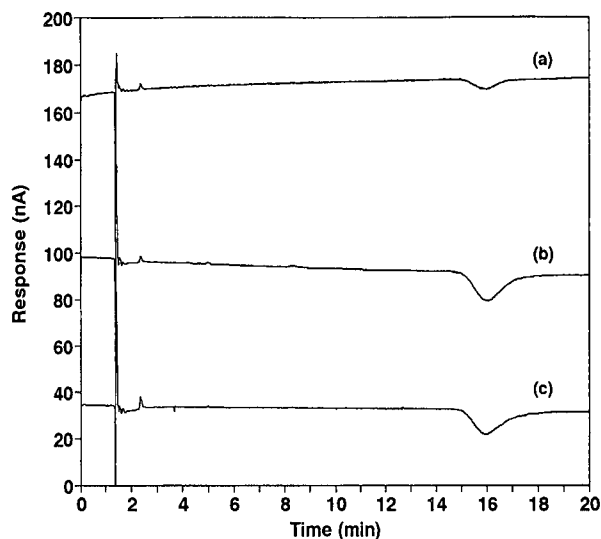


FIGURE 7: Effect of  $t_3$  on ‘oxygen disturbance’ at 16 minutes for a water injection ( $20 \mu\text{l}$ ) with  $E_3 = -200 \text{ mV}$  and  $t_3 =$  (a) 150 ms; (b) 300 ms; (c) 450 ms

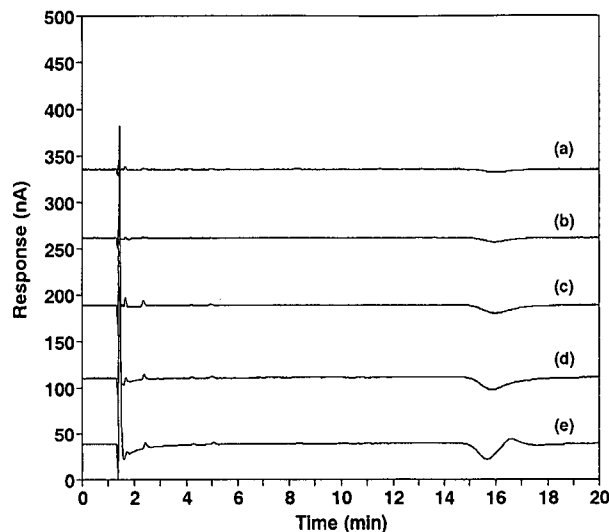
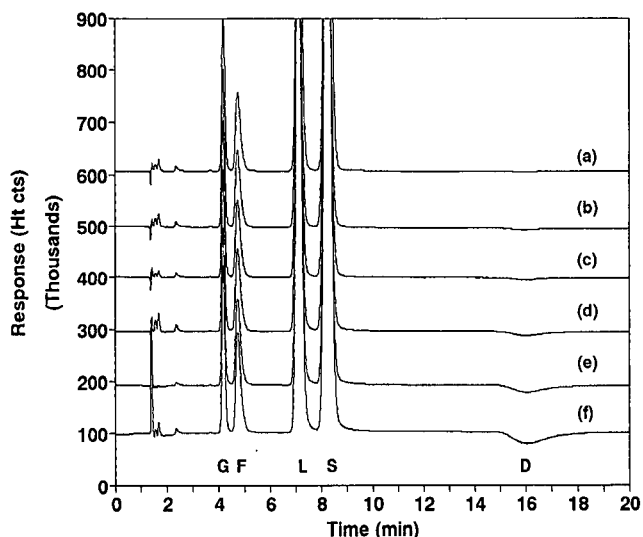


FIGURE 8: Influence of injection volume on ‘oxygen disturbance’ at 16 minutes for a water injection with  $E_3 = -200 \text{ mV}$  and  $t_3 = 450 \text{ ms}$ . (a)  $5 \mu\text{l}$ ; (b)  $10 \mu\text{l}$ ; (c)  $20 \mu\text{l}$ ; (d)  $50 \mu\text{l}$ ; (e)  $100 \mu\text{l}$

•  $AD$  and  $t_1$  – the decay delay and sampling pulse. If, as suggested, the reduction of oxygen occurs during the  $E_3/t_3$  pulse, then increasing the charge decay time [i.e. acquisition delay (AD)] should lessen the effect on the baseline. Aliquots ( $20 \mu\text{l}$ ) of a mixture of glucose, fructose, lactose and sucrose were injected while  $t_1$  and AD were varied. The sampling period and the  $E_2/t_2$  and  $E_3/t_3$  pulses were kept constant. Figure 9 shows the effect on the disturbance, and Table 3 shows the influence on response. Minimum baseline disturbance was evident if  $t_1$  was greater than 500 ms. At this setting response and relative response have almost plateaued. Significant loss in detector response is observed after 700 ms.



**FIGURE 9:** Effect of  $t_1$  on "oxygen disturbance" and sugar response for  $E_3 = -200$  mV;  $t_3 = 450$  ms;  $(t_1-AD) = 200$  ms.  $t_1 =$  (a) 800 ms; (b) 700 ms; (c) 600 ms; (d) 500 ms; (e) 400 ms; (f) 300 ms. (G = glucose; F = fructose; L = lactose; S = sucrose; D = disturbance)

**Table 3**  
Effect of  $t_1$  on detector response (mixed juice S-3 calibration)

$t_1$ (ms)	Response (Hit cts $\times 10^{-2}$ )				Ratio		
	Glucose	Fructose	Lactose	Sucrose	Glucose/ lactose	Fructose/ lactose	Sucrose/ lactose
300	4,29	2,55	17,37	23,63	0,247	0,147	1,360
400	4,04	2,19	14,05	21,44	0,288	0,156	1,526
500	4,05	2,05	12,83	20,08	0,315	0,160	1,566
600	4,03	1,99	12,47	19,72	0,323	0,159	1,581
700	4,04	1,98	12,25	19,42	0,330	0,161	1,585
800	4,09	1,97	12,14	19,09	0,336	0,162	1,572

AD =  $(t_1-200)$  ms;  $E_1 = 50$  mV;  $E_2 = 650$  mV;  $t_2 = 100$  ms;  $E_3 = -200$  mV;  $t_3 = 300$  ms

The cell settings currently in use at the SMRI are shown in Table 4. Potential pulses have been selected so that severe positive or negative voltage excursions are avoided. This should minimise recovery times. Time pulses are long enough to maintain the detector condition, but short enough to avoid too low a sampling frequency. Although the baseline disturbance has almost been removed by these actions, it still remains significant. Hence it is necessary to ensure that the analytical run times selected are such that the disturbance does not influence quantitation. The effect is considerably less marked when using the Dionex STD-PAD-2 cell. The design of the ESA 5040 cell, where the reference is located downstream of the working electrode directly in the constantly changing mobile phase, might be part of the reason.

It has been noticed that frequent polishing of the working electrode is undesirable because of the prolonged stabilisation period. The need for this has become less frequent as better pulsing programmes have been introduced. A tailing fructose peak is often the first indication that the gold electrode surface is not properly conditioned. This effect has occurred less frequently and, in future, it might be possible to re-establish the surface electrochemically (rather than mechanically) by temporarily introducing a more negative  $E_3$ . If placing a solid reference electrode in a constantly changing

matrix does, in fact, contribute to baseline disturbances, this could be seen as a disadvantage of greater importance than the fact that the reference electrode needs no attention. It has been found that the Dionex STD-PAD-2 cell needs little attention other than to replenish the filling solution and to replace the membrane occasionally.

**Table 4**  
Current detector settings in use at the SMRI

Detector setting	Cell	
	Dionex STD-PAD-2 (i)*	ESA 5040 (ii)*
$E_1$ (mV)	50	50
$t_1$ (ms)	420	500
AD (ms)	(100)	300
SP (ms)	200	(200)
$E_2$ (mV)	750	650
$t_2$ (ms)	180	100
$E_3$ (mV)	-200	-200
$t_3$ (ms)	360	300
Range ( $\mu$ A)	3	10

\* Reference: (i) Anon (1993b); (ii) this paper

*Linear range*

*Dionex STD-PAD-2 cell.* During routine use of the published procedure for molasses analysis (Schäffler, 1994) the relative response factors for the three sucrose calibration standards gave relative standard deviation (RSD) values of better than 1% most of the time. On several occasions the precision deteriorated. When this occurred the relative response factor (RRF) for the most concentrated standard had increased, indicating decreasing sucrose response or non-linearity. During the past season the calibration range was decreased by taking 2 ml (rather than 3 ml) aliquots for the final dilution. This gave a working range of 1 000 to 1 500 ng per injection. Although linear response has been more consistent, these levels are still a four-fold increase on those used by Thompson (1990). LaCourse and Johnson (1991) have shown that glucose (transport-controlled oxidation) will produce linear current versus concentration plots over wider concentration ranges than will substances where oxidation is under surface control (e.g. sucrose). They indicated marked deviations from linearity when more than 850 ng of sucrose were injected.

*ESA 5040 cell.* Evaluation of the linear range for this cell (using the adjusted cell settings), indicated that the sucrose response was linear over the range 0 to 1 000 ng ( $r = 0,9979$ ) (Figure 10). However, the linearity necessary for high-precision quantitation dictated that less than 300 ng of sucrose were injected (i.e. solution concentration less than 15 ppm when using 20  $\mu$ l injections) (Figure 11).

*(C): Sample preparation and integrity*

Dilution and filtration (0,45  $\mu$ m cellulose acetate membrane) of the sample has been adequate for most sugar processing samples. Some authors recommend the use of 0,2  $\mu$ m membranes (White and Widmer, 1990; Lamb *et al.*, 1993; Prodoliet *et al.*, 1995) and nylon membranes may be preferable to cellulose acetate (Anon, 1993a). This rapid and relatively inexpensive sample preparation is one of the advantages of HPAEC. However, on occasions, more extensive clean-up may be necessary, e.g. various solid phase extraction cartridges can be used to remove substances such as proteins, phenolic compounds or halides (Anon, 1993a). The in-

clusion of a suitable internal standard (e.g. lactose) allows serial dilutions and multiple manipulations without compromising accuracy. These simple procedures work well with immediate manual injection. However, high throughput routine analyses require automated injection. The possible deterioration of very dilute samples while queuing for analysis has been of some concern. The following procedures have been introduced to overcome these effects in different products.

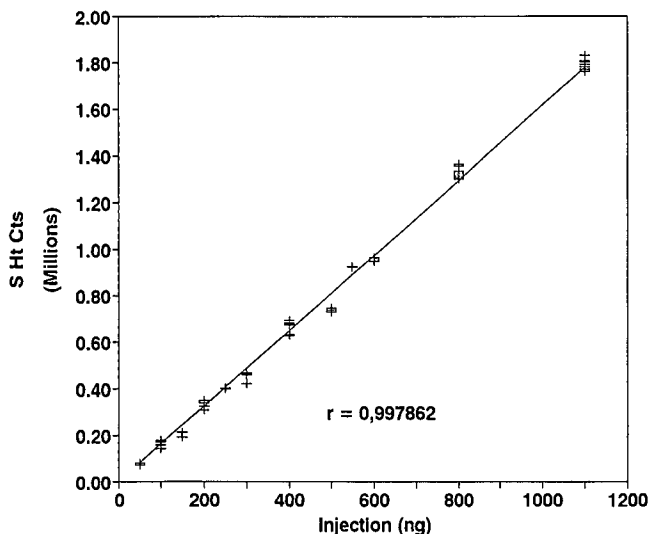


FIGURE 10: Linearity of sucrose response (using ESA 5040 cell)

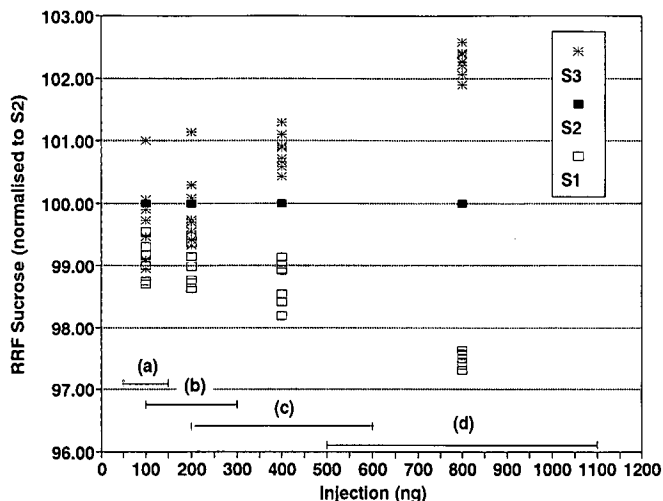


FIGURE 11: Calibration precision for sucrose (using ESA 5040 cell) when injecting (a) 50 to 150 ng; (b) 100 to 300 ng; (c) 200 to 600 ng; (d) 500 to 1100 ng

**Molasses**

The HPAEC analysis of glucose, fructose and sucrose in cane final molasses has been reported to be precise and repeatable. A recent inter-laboratory study involving 11 laboratories (Schäffler, 1994) reported an average  $RSD_r$  for sucrose of 0,7% and average  $RSD_R$  for sucrose of 1,5%. In the same study excellent agreement between results obtained using GC and HPAEC was also reported. On occasion, however, extremely poor precision for duplicate samples has been noticed. This tended to occur more frequently during periods of extremely hot weather. Sample deterioration during long runs was identified as the source of the error. The problem has

been overcome by using sodium azide (0,02%) for the final dilution. Data shown in Table 5 demonstrate the improved precision and repeatability obtained for samples analysed 24 hours after dilution with sodium azide when compared with those diluted with water alone. The standard error (SE) has been reduced from 0,5 to 0,1 while the mean decrease of 0,3 unit is considered acceptable for this analysis, especially since sample delay times are considerably less than 24 hours.

Table 5  
Comparison of sucrose (% on sample) stability for final molasses (FM) samples prepared in water alone or in sodium azide (0,02%)

Sample	Water			Sodium azide (0,02%)		
	Delay time (h)		Difference ( $t_{24}-t_0$ )	Delay time (h)		Difference ( $t_{24}-t_0$ )
	0	24		0	24	
FM 1	28,62	27,64	-0,98	28,92	28,64	-0,28
	28,71	26,07	-2,64	28,78	28,89	0,12
FM 2	28,86	20,80	-8,05	28,58	28,62	0,05
	29,07	28,83	-0,25	28,81	28,84	0,03
FM 3	30,55	30,24	-0,31	30,68	30,43	-0,25
	30,83	30,32	-0,51	30,82	30,47	-0,35
FM 4	31,38	29,25	-2,13	31,50	31,46	-0,04
	31,57	29,49	-2,07	31,60	31,65	0,05
FM 5	31,48	30,61	-0,87	31,78	30,49	-1,29
	31,51	29,60	-1,91	31,63	30,68	0,96
FM 6	30,48	30,55	0,07	30,74	30,48	-0,26
	30,49	30,49	0,01	30,61	30,37	-0,23
FM 7	30,23	29,00	-1,23	30,35	30,31	-0,04
	30,37	29,70	-0,68	30,42	30,17	-0,25
FM 8	26,22	26,51	0,29	26,30	25,67	-0,63
	26,59	26,31	-0,28	26,47	25,85	-0,62
Mean difference			-1,35			-0,31
SD			1,99			0,39
SE			0,50			0,10

**Mixed juice**

Schäffler and Day-Lewis (1992) indicated that excellent agreement could be obtained between sucrose measured by GC or by HPAEC. In that particular study a Dionex SC-PAD-2 cell was used and analyses were carried out *within two hours* of thawing the mixed juice. Despite the small differences for sucrose between GC and HPAEC (mean difference for 17 factories for three weeks of -0,06 unit of sucrose), reservations were expressed that HPAEC sometimes led to underestimation of sucrose from milling tandems by as much as 0,5 unit (personal communication). Although immediate injection of juice samples is obviously the ideal situation, this is usually impractical in the routine analytical environment. Obviously juice deterioration can be extensive and so attempts were made to stabilise the samples awaiting injection. Preparation of mixed juice in sodium azide (0,02%) rather than water led to improvements in analytical reliability (comparable with those noted for molasses) when delays between preparation and injection were unavoidable (Table 6). However, as can be seen in Figure 12, deterioration sometimes continued to increase with time. Samples were then diluted with ethanol (75%) in an attempt to prevent possible enzymic hydrolysis. The final dilution was done automatically just before injection. Results presented in Tables 7 and 8 show that delayed injection of samples preserved with either sodium azide or ethanol gives results comparable with those obtained

<sup>2</sup> Dr BS Purchase, Chairman, Factory Control Advisory Committee, 'New official method for sucrose determination' to all members of FCAC, 6 January 1995.

by GC. The occasional rogue result is still of some concern and further confirmatory work is in progress to establish optimum conditions for autoanalysis of juice samples.

**Table 6**  
Comparison of sucrose (% on sample) stability for mixed juice (MJ) samples prepared in water alone or in sodium azide (0,02%)

Sample	Water			Sodium azide (0,02%)		
	Delay time (h)		Difference (t <sub>24</sub> -t <sub>0</sub> )	Delay time (h)		Difference (t <sub>24</sub> -t <sub>0</sub> )
	0	24		0	24	
MJ 1	7,92	7,92	0,00	7,91	7,99	0,08
	7,98	7,86	-0,12	7,96	7,98	0,02
MJ 2	8,80	8,62	-0,18	8,76	8,58	-0,18
	8,72	8,60	-0,12	8,76	8,69	-0,06
MJ 3	8,67	8,58	-0,09	8,65	8,54	-0,11
	8,29	3,93	-4,36	8,21	8,06	-0,15
MJ 4	8,31	3,86	-4,45	8,21	8,06	-0,15
	8,28	4,02	-4,27	8,26	8,08	-0,19
MJ 5	12,00	12,06	0,06	12,00	12,01	0,01
	12,02	11,92	-0,10	11,97	11,97	0,00
MJ 6	12,02	12,03	0,01	11,90	12,05	0,15
	10,17	10,13	-0,04	10,08	9,98	-0,10
MJ 7	10,17	10,00	-0,17	10,16	10,12	-0,04
	10,11	10,01	-0,11	10,22	10,00	-0,22
MJ 8	10,04	9,50	-0,54	10,09	9,97	-0,12
	10,08	9,67	-0,41	10,12	9,97	-0,15
	10,09	9,57	-0,52	10,09	9,99	-0,10
Mean difference			-0,91			-0,08
SD			1,66			0,10
SE			0,40			0,02

**Sugars**

Tsang *et al.* (1987; 1991) described a direct determination of invert in raw sugars using HPAEC and found that the measurements compared favourably to those obtained by GC (mean difference: GC > HPAEC by 0,016 unit for fructose (at

0,09%) and 0,0007 unit for glucose (at 0,08%)). Morel du Boil and Schäffler (1990) used HPAEC to monitor inversion during refining. A survey of invert levels in cane raw and white sugars indicated that the procedure was precise (mean RSD of 3,2% for glucose and 4,1% for fructose for 18 samples with glucose or fructose in the range 20 to 3 000 ppm) (Morel du Boil, 1994). However, it was noticed that glucose and fructose levels could increase significantly if delays occurred between sample preparation and sample injection. The addition of 0,02% sodium azide to prevent microbial spoilage gave no improvement when an unaffinated raw sugar was analysed over a 24 hour period (Table 9).

Any chemical inversion of sucrose would have a large influence on the low levels of glucose and fructose in high purity products. To prevent such chemical inversion a white and an affinated raw sugar were dissolved in sodium azide at different pH levels. The solution pH had no effect on the response for glucose or fructose standards and so epimerisation effects can be regarded as insignificant (Table 10). Only samples prepared in sodium azide (0,02%) adjusted to pH 10,4 showed acceptable stability over a 24 hour period. The data for glucose are shown in Table 11. The trend for fructose was similar. Clearly the affinated raw sugar showed more rapid increases in glucose and fructose than did the white sugar. This increase was also greater than theoretically predicted and might possibly be attributed to catalytic effects associated with the ash constituents of raw sugar. Homogeneous samples (sub-samples of syrup prepared from sugar) and the incorporation of lactose as internal standard gave improved precision (RSD of 1,2% for glucose or fructose for 17 samples with glucose or fructose between 200 and 1 500 ppm). The minimum detectable quantity of glucose or fructose was about 1 ng when using sugar concentrations of about 0,5% and 20 µl injections (Morel du Boil, 1994).

The causes of increased glucose and fructose during analysis of high purity products have not been adequately explained in terms of either microbial activity or chemical inversion. However, it appears that, provided samples are

**Table 7**  
Sucrose (%) in mixed juice: comparison of GC with HPAEC using sodium azide as preservative

Sample	GC				HPAEC				Difference (GC-HPAEC)
	Rep 1	Rep 2	Rep 3	Mean	Rep 1	Rep 2	Rep 3	Mean	
MJ 1	12,02	11,90	11,93	11,95	11,91	11,83	11,78	11,84	0,11
MJ 2	12,95	12,99	12,97	12,97	12,92	12,90	12,91	12,91	0,06
MJ 3	9,09	9,13	9,08	9,10	9,13	9,09	9,16	9,13	-0,03
MJ 4	12,35	12,36	12,34	12,35	12,34	12,33	12,39	12,35	0,00
MJ 5	12,32	12,32	12,30	12,31	12,29	12,24	12,27	12,27	0,05
MJ 6	10,43	10,45	10,42	10,43	10,43	10,42	10,18	10,34	0,09
MJ 7	10,03	10,02	10,04	10,03	10,11	10,01	10,18	10,10	-0,07
MJ 8	12,02	11,90	11,93	11,95	11,89	11,99	11,97	11,95	0,00
MJ 9	12,95	12,99	12,97	12,97	12,95	12,96	13,03	12,98	-0,01
MJ 10	9,09	9,13	9,08	9,10	9,13	9,14	9,45	9,24	-0,14
MJ 11	12,35	12,36	12,34	12,35	12,44	12,49	12,55	12,49	-0,14
MJ 12	12,32	12,32	12,30	12,31	12,42	12,38	12,37	12,39	-0,08
MJ 13	10,43	10,45	10,42	10,43	10,67	10,48	10,23	10,46	-0,03
MJ 14	10,03	10,02	10,04	10,03	10,10	10,07	10,07	10,08	-0,05
MJ 15	12,02	11,90	11,93	11,95	11,88	11,81	11,78	11,82	0,13
MJ 16	12,95	12,99	12,97	12,97	13,47	13,32	13,18	13,32	-0,35
MJ 17	9,09	9,13	9,08	9,10	9,13	9,02	8,98	9,04	0,06
MJ 18	12,35	12,36	12,34	12,35	12,30	12,22	12,33	12,28	0,07
MJ 19	12,32	12,32	12,30	12,31	12,30	12,44	12,19	12,31	0,00
MJ 20	10,43	10,45	10,42	10,43	10,52	10,74	10,54	10,60	-0,17
MJ 21	10,03	10,02	10,04	10,03	10,09	10,17	10,12	10,13	-0,10
Mean									-0,03

**Table 8**  
**Sucrose (%) in mixed juice: comparison of GC with HPAEC using 75% ethanol as preservative**

Sample	GC				HPAEC				Difference (GC-HPAEC)
	Rep 1	Rep 2	Rep 3	Mean	Rep 1	Rep 2	Rep 3	Mean	
MJ 1	10,98	10,99	11,02	11,00	10,87	10,83	10,69	10,80	0,20
MJ 2	11,18	11,23	11,23	11,21	11,16	11,06	11,11	11,11	0,10
MJ 3	8,88	8,87	8,88	8,88	9,02	8,93	9,00	8,98	-0,11
MJ 4	11,24	11,24	11,27	11,25	11,17	11,25	11,31	11,24	0,01
MJ 5	11,83	11,80	11,78	11,80	11,58	11,71	11,74	11,68	0,13
MJ 6		9,53	9,63	9,58	9,57	9,59	9,63	9,60	-0,02
MJ 7	8,58		8,51	8,55	8,59	8,54	8,69	8,61	-0,06
MJ 8	10,32	10,39	10,29	10,33	10,23	10,33	10,25	10,27	0,06
MJ 9	11,67	11,55	11,59	11,60	11,60	11,52	11,58	11,57	0,04
MJ 10	9,37		9,40	9,39	9,48	9,44	9,46	9,46	-0,08
MJ 11	10,80	10,84	10,81	10,82	10,88	10,94	10,92	10,91	-0,10
MJ 12	12,00	11,96	11,97	11,98	12,01	11,91	12,00	11,97	0,00
MJ 13	9,61	9,68	9,65	9,65	9,66	9,70	9,71	9,69	-0,04
MJ 14	9,42	9,36	9,36	9,38	9,43	9,41	9,37	9,40	-0,02
MJ 15	9,58	9,58	9,59	9,58	9,61	9,63	9,58	9,61	-0,02
MJ 16	10,90	10,79	10,80	10,83	10,91	10,85	10,75	10,84	-0,01
MJ 17	9,28	9,19	9,21	9,23	9,37	9,29	9,29	9,32	-0,09
MJ 18	11,60	11,65	11,59	11,61	11,40	11,58	11,52	11,50	0,11
MJ 19	12,21	12,24	12,26	12,24	11,97	11,93	11,95	11,95	0,29
MJ 20	10,22	10,18	10,23	10,21	10,24	10,11	10,14	10,16	0,05
MJ 21	9,19	9,17	9,26	9,21	9,27	9,24	9,25	9,25	-0,05
MJ 22	7,75	7,76	7,84	7,78	7,67	7,75	7,84	7,75	0,03
MJ 23	8,92	8,95	8,92	8,93	8,89	8,84	8,94	8,89	0,04
MJ 24	7,84	7,81	7,82	7,82	7,74	7,81	7,84	7,80	0,03
MJ 25	10,58	10,62	10,59	10,60	10,52	10,48	10,61	10,54	0,06
MJ 26	9,89	9,84	9,86	9,86	9,88	9,88	9,87	9,88	-0,01
MJ 27	9,39	9,38	9,38	9,38	9,40	9,38	9,39	9,39	-0,01
MJ 28	8,45	8,44	8,43	8,44	8,45	8,50	8,49	8,48	-0,04
MJ 29	9,81	9,84	9,81	9,82	9,82	9,94	9,92	9,89	-0,07
MJ 30	9,93	9,85	9,84	9,87	9,96	9,95	9,96	9,96	-0,08
MJ 31	8,63	8,59	8,60	8,61	8,81	8,80	8,78	8,80	-0,19
MJ 32	11,19	11,15	11,12	11,15	11,31	11,38	11,32	11,34	-0,18
MJ 33	10,50	10,52	10,52	10,51	10,53	10,49	10,39	10,47	0,04
MJ 34	9,79	9,76	9,82	9,79	9,82	9,82	9,82	9,82	-0,03
MJ 35	8,89	8,88		8,89	8,97	8,90	8,89	8,92	-0,03
Mean									-0,06

**Table 9**

**Effect of 0,02% sodium azide on the analysis of glucose and fructose in unaffinated raw sugar**

Delay time (h)	Water				Sodium azide			
	Glucose (ppm)		Fructose (ppm)		Glucose (ppm)		Fructose (ppm)	
	0	24	0	24	0	24	0	24
	1350	1675	1604	1980	1380	1755	1653	2060
		1650		1990		1810		2125
		1725		2060		1800		2110
Mean	1379	1685	1638	2010	1403	1790	1679	2100
RSD (%)		2,3		2,2		1,6		1,6

diluted with alkaline sodium azide, the effect can be prevented. The use of large enough samples to ensure homogeneity, together with the addition of an internal standard, gives acceptable analytical precision. The precautions will be included in a proposed ICUMSA evaluation for determining trace monosaccharides in high purity sugars.

**Table 10**

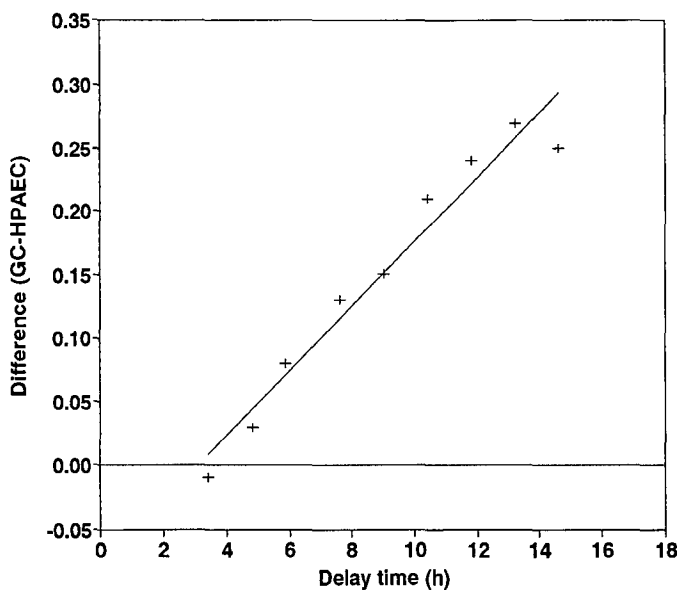
**Influence of solvent pH on glucose and fructose response**

Solvent	Glucose			Fructose		
	Response (Ht cts/mg/l) (x 10 <sup>-6</sup> )	Mean	RSD (%)	Response (Ht cts/mg/l) (x 10 <sup>-6</sup> )	Mean	RSD (%)
Water	1,22 1,21	1,22	1,06	1,010 0,997	1,004	0,86
(a)*	1,17 1,22	1,20	2,59	0,977 1,032	1,004	3,91
(b)	1,16 1,15	1,15	0,50	0,958 0,940	0,949	1,30
(c)	1,22 1,19	1,20	1,87	1,010 0,994	1,002	1,10
Overall		1,19	2,49		0,990	3,04

\* (a) Sodium azide (0,02%) - pH 6,9  
 (b) Sodium azide (0,02%) - pH 8,9  
 (c) Sodium azide (0,02%) - pH 10,4

**Table 11**  
Effect of dilution solvent on increase in glucose (ppm) when analysis of refined or affinated raw sugar is delayed

Delay (h)	Water		Sodium azide (0,02%)		Sodium azide (0,02 % @ pH 9)		Sodium azide (0,02% @ pH 10,4)	
	Refined	Affinated raw	Refined	Affinated raw	Refined	Affinated raw	Refined	Affinated raw
5	3	35	2	70	3	18	1	3
8	5		0		4		2	
22	15	82	17	270	28	60	6	2
26	18	91	7	365	32	133	10	8



**FIGURE 12:** Effect of injection delay on sucrose determination in mixed juice – difference between GC and HPAEC [using sodium azide (0,02%) as diluent]

### Conclusions

The fine-tuning procedures described in this paper have contributed to improved precision and reliability.

- Temperature has a marked effect on the determination of sucrose in factory products. It is important that both the column and the detector are thermostatically controlled (at 27 to 28°C) for precise and accurate results. Control of the laboratory temperature (at 22 to 23°C) assists in this control.
- The potential-time pulsing programmes for the electrochemical detectors were optimised to avoid possible interferences from both PAD responsive non-sugars and from dissolved oxygen. In addition, less severe conditioning settings have resulted in steadier operation with less frequent detector maintenance. The fructose peak is a useful monitor of the detector condition.
- The concentrations previously used for molasses have been decreased to ensure that sucrose linearity is better than 1% relative to the three calibration standards.

- The addition of sodium azide (0,02%) to cane molasses solutions has overcome the problem of sample deterioration when samples queue for automated analysis. Although sodium azide appeared equally effective for mixed juice analyses, the occasional rogue result is still of some concern. Samples diluted with either sodium azide or with ethanol (75%) gave results that were comparable to those obtained using the official GC procedure. Work is in progress to establish the optimum conditions for automated analysis of juice samples.
- Because of the extremely low levels of invert in raw and white sugars, even low inversion rates (chemical, enzymic or microbial) during delays will have a significant effect on invert concentrations. Dilute solutions of raw and white sugars were not adequately preserved in sodium azide alone. Acceptable stability was obtained when samples were prepared in sodium azide adjusted to pH 10.
- During the 1995-96 season the routine analytical programme for molasses required over 4 000 injections. The system operated well within targeted limits throughout the season with an average linearity RSD for sucrose calibration of 0,86%. Regular column clean up and occasional replacement of the guard column has resulted in over 13 000 injections for the current column without any discernible deterioration in performance.

### REFERENCES

- Andrews, RW and King, RM (1990). Selection of potentials for pulsed amperometric detection of carbohydrates at gold electrodes. *Analyt Chem* 62: 2130-2134.
- Anon (1989). Analysis of carbohydrates by anion exchange chromatography with pulsed amperometric detection. *Dionex Tech Note* 20: 23 pp.
- Anon (1993a). Analysis of carbohydrates by high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD). *Dionex Tech Note* 20(7/93): 12 pp.
- Anon (1993b). Optimal settings for pulsed amperometric detection of carbohydrates using Dionex pulsed electrochemical and amperometric detectors. *Dionex Tech Note* 21(6/93): 3 pp.
- Anon (1994). CouloChem II Operating Manual. ESA Inc., 22 Alpha Road, Chelmsford, MA 01824, USA.
- Anon (1996). Collaborative study report approved by the Methods Committee, Association of Official Analytical Chemists: 'Sugars in beet and cane final molasses', Ion chromatographic method. Jill Jekot, Dionex Corp, PO Box 3603, Sunnyvale, CA 94088-3063, USA.
- Bowers, ML (1991). A new analytical cell for carbohydrate analysis with a maintenance-free reference electrode. *J Pharm and Biomed Analysis* 9(10-12): 1133-1137.
- Corradini, C, Cristalli, A and Corradini, D (1993). High performance anion-exchange chromatography with pulsed amperometric detection of nutritionally significant carbohydrates. *J Liq Chromatogr* 16(16): 3471-3485.
- Johnson, DC and LaCourse, WR (1990). Liquid chromatography with pulsed amperometric detection at gold and platinum electrodes. *Analyt Chem* 62(10): 589A-597A.

- LaCourse, WR and Johnson, DC (1991). Optimisation of waveforms for pulsed amperometric detection (pad) of carbohydrates following separation by liquid chromatography. *Carbohydr Res* 215: 159-178.
- LaCourse, WR and Johnson, DC (1993). Optimisation of waveforms for pulsed amperometric detection of carbohydrates based on pulsed voltammetry. *Analyt Chem* 65: 50-55.
- Lamb, JD, Myers, GS and Edge, N (1993). Ion chromatographic analysis of glucose, fructose and sucrose in raw and processed vegetables. *J Chromatogr Sci* 31: 353-357.
- Morel du Boil, PG (1994). The high performance anion exchange chromatographic (HPAEC) measurement of glucose and fructose in sugar crystals and similar very high purity products. *Sugar Milling Res Inst Tech Rep* No 1697: 14 pp.
- Morel du Boil, PG and Schäffler, KJ (1990). Ion chromatography: A comparison between anion and cation exchange HPLC for carbohydrates. *Proc Sugar Processing Res Inst* pp 397-413.
- Prodolliet, J, Bugner, E and Feinberg, M (1995). Determination of carbohydrates in soluble coffee by anion-exchange chromatography with pulsed amperometric detection: Interlaboratory study. *J Ass of Official Analyt Chemists International* 78 (3): 768-782.
- Schäffler, KJ (Referee) (1994). Subject 8: Chromatographic techniques for sugars. *Proc Intern Comm for Uniform Methods of Sugar Analysis (ICUMSA)* 21: 282-315.
- Schäffler, KJ and Day-Lewis, CMJ (1983). A quality assurance programme for the weekly analysis of sugars in cane final molasses by gas chromatography. *Proc S Afr Sug Technol Ass* 57: 33-37.
- Schäffler, KJ and Day-Lewis, CMJ (1992). Can HPLC replace GC as a viable, accurate, routine procedure for determining carbohydrates in sugar cane juices and factory products? *Proc Sug Processing Res Inst* pp 191-206.
- Thielecke, K (Referee) (1994). Subject 2: Oligosaccharides and polysaccharides. *Proc Intern Comm for Uniform Methods of Sugar Analysis (ICUMSA)* 21: 165-217.
- Thompson, JC (1990). Methods for the determination of carbohydrates by ion chromatography. *Proc Sug Processing Res Inst* pp 381-395.
- Tsang, WSC, Bengtsson, M, Tjebbes, J and Clarke, MA (1991). Ion chromatography, flow injection analysis and other techniques for the future. *Zuckerind* 116: 42-47.
- Tsang, WSC, Clarke, MA, Godshall, MA and Valdes, MM (1987). Raw sugar analysis by chromatographic methods. *Proc Sug Ind Technol* 46: 27-42.
- White, DR and Widmer, WW (1990). Application of high performance anion-exchange chromatography with pulsed amperometric detection to sugar analysis in citrus juices. *J Agric Food Chem* 38: 1918-1921.