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ANALYSIS OF SUGARS IN FINAL MOLASSES BY ION CHROMATOGRAPHY

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Abstract

Previous work at the Institute has shown that cation exchange HPLC with refractive index detection produced inflated results for fructose, glucose and sucrose in final molasses. Anion exchange chromatography with electrochemical detection offers a procedure that is both more selective and specific than conventional high performance liquid chromatography (HPLC). The precision and accuracy of this technique are compared with the official gas chromatography (GC) procedure for final molasses.

Introduction

High pressure liquid chromatography (HPLC) was developed in the early 1970s. The SMRI obtained its first instrument in 1977 and five years were spent attempting to develop a reliable method for the analysis of sugars in final molasses. Amino-bonded silica columns were initially used for carbohydrate analysis as they were capable of separating the three sugars. Unfortunately the amino group reacted with reducing sugars and indifferent results (Kort, 1980) were obtained. In addition, the eluant, acetonitrile, was both expensive and toxic.

Advances in instrumentation and column technology in the mid-1980s rekindled interest in this procedure. An HPLC system, acquired by the Sugar Milling Research Institute (SMRI) in 1987, was used to separate carbohydrates on cation exchange columns, usually in the calcium or sodium forms. These columns had the advantage that the eluant was basically water and separations were therefore carried out safely and cheaply. Back pressures were relatively low (200-800 psi), resulting in a change of name from high 'pressure' to high 'performance' liquid chromatography. In 1989 the Institute purchased an automated, state-of-the-art, liquid chromatograph. The improvement in analytical precision was dramatic (Day-Lewis, 1989). Several cation exchange columns were evaluated for the analysis of sugars in a wide range of factory products. Details of these studies were published recently (Day-Lewis and Schäffler, 1990). Satisfactory results were obtained for all products with the exception of final molasses. Glucose and sucrose data were marginally higher than gas chromatography (GC) results but fructose was significantly higher (approximately 0,8 units). These inflated values were attributed to co-eluting impurities. Unfortunately removal of these impurities was not successful.

In all the above work, a refractive index (RI) detector was used to determine sugar concentrations. This detector is in principle non-specific and it is not surprising therefore that co-eluting impurities inflated sugar estimates.

In the early 1980s the Dionex Corporation introduced an anion exchange (AE) column for the separation of carbohydrates (Anon., 1987). The separation mechanism of these columns relies on the fact that carbohydrates are complex alcohols that can be ionised at high pH. The acidity of each hydroxyl group depends on neighbouring groups and different sugars have different degrees of dissociation at certain pH values. With an eluant at the appropriate pH, many sugars can be separated rapidly by high performance ion chromatography (HPIC) (Anon., 1989). As the ionisation of sugars is pH sensitive, these columns are notably more selective than the gel-based cation exchange columns.

In 1983 Dionex introduced an electrochemical detector to monitor sugar separations. Carbohydrates can be oxidised readily on the surface of a gold electrode. Most sugars produce a maximum current at similar applied potentials. This is important since the pulsed amperometric detector (PAD) is equally efficient at detecting reducing (fructose and glucose) and non-reducing (sucrose) sugars. A repeating sequence of three applied potentials for specific durations ensures that the surface of the gold electrode remains in its original state even after prolonged use. The PAD, besides being more sensitive, is therefore more specific than the refractive index detector.

Morel du Boil (1991) first used the Dionex AE/PAD system at the Institute to confirm that theanderose, a trisaccharide, was present in cane products and that it was partially responsible for sucrose crystal elongation. Morel du Boil and Schäffler (1990), participating in an ICUMSA collaborative study, used the same technique to measure sucrose, fructose and glucose in C-molasses samples. Thompson (1990) improved the precision of the AE/PAD procedure by introducing lactose as an internal standard. This approach ensured that multiple dilutions had no serious effect on the method's precision. He also corrected for the PAD's drift by bracketing samples with calibration standards.

This paper describes the use of HPIC for the analysis of sugars in final molasses and compares the results with those of the official GC method (Anon., 1985).

Method and materials

Equipment

A Spectra-Physics SP 8880 autosampler and Spectra-Physics IsoChrom pump were coupled to a Dionex CarboPac PA1 guard and main columns using sodium hydroxide (0,15M) as eluant. A Dionex pulsed amperometric detector and Hewlett-Packard 3396A integrator were used to monitor column eluants. Separations were carried out at constant temperature. Two different types of flow-through cells were used with the PAD, the original aqueous cell and a solvent compatible cell.

Mobile phase

Correct preparation of the mobile phase is critical if contamination from sodium carbonate is to be avoided. Experimental details are outlined in Appendix 1. Operating flowrate was 1 cm^3 per minute. The flow was reduced to 0,1 cm^3 per minute when the column was not in use. Column eluant was not recycled.

Sample preparation

Details for the preparation and running of samples are supplied in Appendix 2.

Results and discussion

Detector flow-through cells

Dionex provides users with two different cells for use with the PAD controller. They are:

(a) Aqueous Cell (Cell 1). This cell can only be used in water-based solutions. In addition, the reference electrode is separated from the rest of the cell by a semi-permeable membrane. This membrane and the electrolyte need to be replaced periodically.

(b) *Solvent compatible cell (Cell 2).* This cell can also be used with organic solvents. The silver/silver chloride electrode is encapsulated in glass and therefore does not require regular maintenance. However, if the electrode is damaged, replacement costs are high.

Both cells were tested for their suitability for sugar analysis. The results of this study are summarised in Table 1 which indicates that precision and linear range were similar. Cell 2 is approximately nine times more sensitive than cell 1, due possibly to the lower cell volume and/or the different reference electrode. For this reason, molasses samples need to be diluted to 0,01% rather than 0,03% prior to injection into the HPIC system, and the range increased from 10 000 to 30 000 nA. The sensitivity of the PAD can be gauged from the large dilutions required. Dilution errors are avoided by adding lactose as an internal standard prior to serial dilution.

Table 1

Comparison of PAD flow-through cells for the separation of carbohydrates

Description	Cell 1	Cell 2
Cell type	water only	organics
Dionex part number	36 276	42 300
Cost (Rand)	6 300	8 320
Electrode replacement cost	nil	880
Cell volume (μl)	9,5	3,5
PAD range (nA) (Optimum)	10K	30K
Linear range (ppm, sucrose)	2–100	0,5–50
Molasses concentration %	0,03	0,01
Precision*	0,3–1,0	0,4–0,9

* Precision is defined as the RSD of mean peak heights from 10 consecutive injections of a molasses standard.

Standardisation has been made on cell 1 as it is cheaper than cell 2 (capital and running costs). Contrary to publications from Dionex, it was found that the reference electrode needed very little attention.

Precision

GC is currently used by the South African sugar industry to measure sugars in mixed juice and final molasses. If a new HPIC procedure were to replace GC then the precision of the new method must be as good as that produced by the official GC method. Precision can be expressed in several different ways. Many laboratories today use the repeatability concept (Anon., 1990; Mellet *et al.*, 1982; Schäffler and Day-Lewis, 1983; Thompson, 1990).

Thompson (1990) statistically evaluated the precision of a similar procedure using a Dionex column and PAD system. Three possible sources of error were investigated:

(a) The variability of the HPIC itself. This was tested by looking at the difference between duplicate injections.

(b) The scatter derived from the analyst performing the various operations (weighing, additions, mixing, dilutions etc.). This was examined by monitoring the precision of molasses duplicates from individual weighings.

(c) Fluctuation due to changes in laboratory conditions and calibration control factors. This was investigated by scrutinising results from identical samples analysed on different days.

This approach was repeated at the Institute on six cane molasses samples. The local results and those determined by Thompson (1990) can be found in Table 2. A study of the data in this table indicates that:

(a) The recent precision tests at the SMRI are in agreement with those reported by Thompson. Repeatabilities were similar for all three sugars.

(b) The SMRI and Thompson obtained repeatabilities (0,3 and 0,2 respectively) that rival the 0,4 unit quoted by Mellet *et al.* (1982) for direct pol. This pol analysis has always been considered to be a highly precise procedure.

(c) The precision for the two monosaccharides was also excellent (r = 0,05 to 0,10).

(d) The precision of the official GC procedure was also determined by taking results randomly from the routine weekly figures. The HPIC repeatabilities are arguably better than those derived from the GC determinations.

(e) As the repeatability data listed in Table 2 are similar for all treatments, it would appear that the overall precision of the determination is limited by short term variability of the HPIC system. As increased scatter in quantitative data was observed when the air conditioner was switched off, it is necessary to house the system in an air conditioned room. It is also extremely important to avoid carbon dioxide ingress to the solvent as this will result in drift of detector response. A third source of variability was ageing of the rotor seal in the Rheodyne injection valve. This should be replaced periodically. Finally, since detector drift was found to increase as fouling of the gold electrode occurred, the surface was polished periodically. No other maintenance has been required.

Table 2

Comparison of repeatabilities (r) (r = 2,28 X standard deviation)

Source of error	SMRI			Thompson		
	Sucrose	Glucose	Fructose	Sucrose	Glucose	Fructose
Inj 1 vs Inj 2	0,33	0,04	0,08	0,24	0,13	0,18
Rep 1 vs Rep 2	0,32	0,11	0,08	0,20	0,14	0,15
Day 1 vs Day 2	0,25	0,07	0,10	0,14	0,07	0,19
Mean	0,30	0,07	0,09	0,19	0,11	0,17
GC*	0,36	0,12	0,15			

* GC SMRI precision results were obtained under similar but not identical conditions

Accuracy

The accuracy of a new procedure is always difficult to establish. It is therefore fortunate that the new HPIC procedure could be compared with:

(a) Results from an international collaborative study (Dutton, 1990). The six molasses samples used in the 1990 ICUMSA ring test had been kept frozen and were analysed on four separate occasions using the new HPIC procedure. Comparisons with ICUMSA GC and HPLC data are included in Table 3.

Table 3

Comparison of HPIC results (SMRI) with ICUMSA interlaboratory data for GC and HPLC (Subject 9, 1990)

Sample	A	B	C	D	E	F	Mean
Sucrose							
SMRI (1)	30,6	29,3	29,9	32,0	34,8	33,0	31,6
SMRI (2)	30,9	29,0	29,8	32,0	35,0	33,1	31,6
SMRI (3)	30,7	29,3	30,1	32,3	35,2	33,1	31,8
SMRI (4)	30,7	29,1	30,0	32,1	35,0	33,1	31,7
Ave SMRI	30,7	29,2	30,0	32,1	35,0	33,1	31,7
ICUMSA GC	30,5	29,4	30,1	32,3	35,4	33,4	31,9
ICUMSA LC	30,8	29,6	30,4	32,3	35,5	33,2	32,0
Glucose							
SMRI (1)	3,6	3,6	4,6	2,5	3,2	3,6	3,5
SMRI (2)	3,6	3,6	4,6	2,4	3,1	3,5	3,5
SMRI (3)	3,6	3,6	4,6	2,4	3,1	3,5	3,5
SMRI (4)	3,6	3,6	4,7	2,4	3,1	3,5	3,5
Ave SMRI	3,6	3,6	4,6	2,4	3,1	3,5	3,5
ICUMSA GC	3,6	3,7	4,7	2,6	3,2	3,6	3,6
ICUMSA LC	3,6	3,7	4,7	2,6	3,2	3,5	3,6
Fructose							
SMRI (1)	6,2	7,2	7,8	5,4	5,1	4,3	6,0
SMRI (2)	6,2	7,2	7,8	5,4	5,1	4,3	6,0
SMRI (3)	6,1	7,2	7,8	5,5	5,1	4,3	6,0
SMRI (4)	6,1	7,1	7,8	5,4	5,1	4,4	6,0
Ave SMRI	6,2	7,2	7,8	5,4	5,1	4,3	6,0
ICUMSA GC	6,1	7,2	7,8	5,5	5,1	4,3	6,0
ICUMSA LC	6,2	7,3	7,9	5,5	5,2	4,3	6,1

Agreement between the HPIC results and the ICUMSA data was excellent, any small differences being within the experimental error of the various determinations. This was indeed gratifying as previous HPLC procedures did not always produce good agreement for all three sugars.

(b) The official GC Procedure (Anon, 1985). The HPIC system, with cell 2 installed, was used to analyse all the weekly molasses samples (n=50) from three consecutive weeks in September 1991. The raw data for each mill appear in Appendix 3. The data were subjected to t-test comparisons to determine whether the differences between GC and HPIC were statistically significant for each sugar. Statistical results have been summarised in Table 4. The differences were statistically significant for all three sugars, even though the mean (GC-HPIC) differences were only -0,08, +0,07 and -0,20 units for fructose, glucose and sucrose respectively. This rather surprising anomaly can possibly be ascribed to the fact that the precision of the GC and HPIC procedures is excellent. This results in a low value of SD (GC-LC) and hence a high calculated t-value. The confidence intervals for the two methods are therefore extremely narrow and statistically different even though for all practical purposes the two procedures produced identical results (Wernimont, 1987).

It must also be remembered that every analytical technique has inherent systematic error or bias. The minor differences observed for the weekly molasses results are probably due to small biases in both procedures.

Table 4

Statistical comparison between GC and HPIC for the analysis of sugars in 50 weekly molasses samples (Null hypothesis H0: Mean difference = 0)

	Fructose		Glucose		Sucrose	
	GC	LC	GC	LC	GC	LC
Mean	6,55	6,63	3,97	3,90	31,1	31,3
SD between 6 samples	1,33	1,31	1,42	1,40	1,61	1,56
Minimum	4,00	4,10	1,90	1,90	26,1	26,4
Maximum	11,1	11,0	9,9	9,7	36,5	36,3
Difference (GC-LC)	−0,07		+0,07		−0,20	
SD (GC-LC)	0,09		0,08		0,19	
t-value calculated from above	−5,97		+6,10		−7,51	
t-value for 99,9% confidence level	3,505		3,505		3,505	
Reject/accept H0:	Reject		Reject		Reject	

Operation

To date more than 3 000 injections have been carried out with no build up of back pressure and no loss of column efficiency. This point is extremely important as previous columns tested all tended to deteriorate after 1 000-2 000 injections. As columns are expensive, the longer life of the AE columns means real savings in operating costs. When the column was badly contaminated during the analysis of unknown contract samples (see Figure 1A), it was successfully regenerated with 1M acetic acid followed by 1M sodium hydroxide (see Figure 1B). This robustness is an added advantage of AE columns. The highly alkaline eluant ensures that there is no bacterial growth in the system.

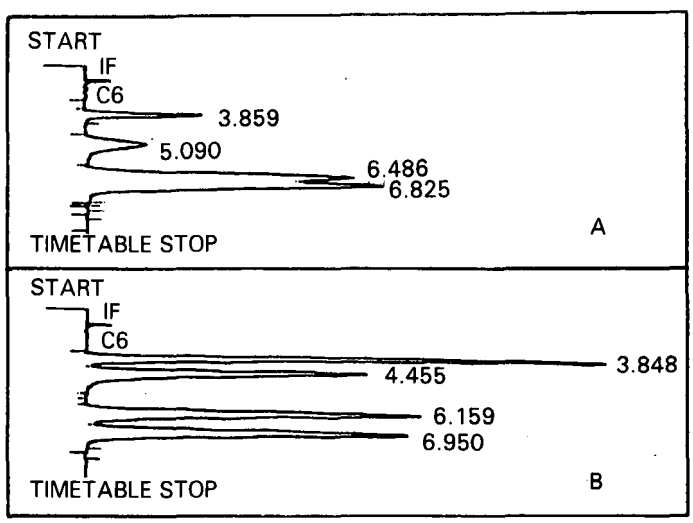

FIGURE 1 Separation of sugars by AE/PAD, elution order from top to bottom being glucose, fructose, lactose (internal standard) and sucrose. (A = after contamination, B = after regeneration).

Analysis time

If HPIC is to replace GC then it is desirable that the total run time should be shorter than that required for GC. A comparison of run times for a typical weekly molasses run (17 factories in duplicate) is given in Table 5. It is obvious from this table that sugar results could be available to sugar factories a day earlier if HPIC rather than GC were used. This time advantage can be especially useful at month-ends when deadlines for factory figures are critical.

Table 5

Comparison of GC and HPIC analysis times for 17 molasses samples (time in hours)

	Sample preparation	Dead time	Chromat-ography	Total
GC	8	24	17	49
HPIC	4	0	14	18

Conclusions

The current system with Dionex anion exchange guard and main columns and PAD detection with either of the two cells is ideally suited for the determination of sugars in molasses. It compares favourably with GC and with published literature with respect to precision and accuracy. This method will be tested during the 1992/93 season for the sugars in Direct Analysis of Cane (DAC) and mixed juice samples. Current indications suggest that AE/PAD will replace GC for routine sugar analysis in the foreseeable future.

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APPENDIX 1

Preparation of mobile phase for anion exchange chromatography

It is essential that air (i.e. CO_2) be excluded at all times. Therefore sparging and blanketing the mobile phase with helium is necessary at ALL stages of eluant handling or preparation. Pyrex storage vessels seem to be superior to plastic or polythene containers.

Preparation of 50% NaOH stock solution

BDH Analar NaOH pellets (Product No 10252) and water from a Waters reverse osmosis (RO) unit are used to prepare the stock solution. Generally 300 g of 50% stock are prepared at any one time. Procedure:

(a) Filter the RO water (0,45μ membrane filter).

(b) Transfer 150 ml of filtered RO water into a 250 ml Schott screw cap bottle containing a magnetic stirrer.

(c) Stir and sparge with helium for at least 30 minutes.

(d) Weigh 150 g Analar NaOH pellets (just before use).

(e) Add the pellets to the sparged water fairly rapidly and continue sparging and stirring until dissolved.

(f) Allow solution to cool slightly. Remove the stirrer and sparger and close bottle.

(g) Leave the stock to stand undisturbed for at least 5 to 7 days before use.

(h) Do not disturb the solution at any time. Always blanket solution with helium when opened. Pipette carefully from the top clear portion. Close the bottle immediately after use.

Preparation of working eluants

It is again essential to exclude CO_2 at all times. It is important to sparge well with helium. On no account should used solvent be recycled.

(a) Filter the RO water.

(b) Place the required volume (e.g. 2 litres) in a Pyrex bottle fitted with a screw cap containing three small holes (i.e. for the helium sparging tube, the eluant outflow line and a vent to ensure constant blanketing without build-up of pressure). Sparge with helium for about 30 minutes.

(c) Pipette 15,6 ml NaOH stock into 2 litres of the sparged solvent to prepare 150 mM NaOH eluant. Ensure that the solvent is sparged continuously.

(d) Do not use more than about two-thirds to three-quarters of the stock sodium hydroxide. Decreasing retention times are a good indication that CO_2 contamination has occurred. It is then necessary to prepare fresh working solution and/or stock solution.

APPENDIX 2

Procedures for preparing calibration standards and molasses samples

Lactose solution (internal standard)

Weigh 8,000 g of lactose into a 100 ml beaker. Dissolve and make to the mark in a 250 ml volumetric flask. Store the stock solution in a refrigerator. Allow the stock solution to reach room temperature before use.

Standards

Preparation of the three calibration standards is shown in the Table below.

	S1	S2	S3
Glucose (g)	0,020	0,060	0,100
Fructose (g)	0,030	0,070	0,110
Sucrose (g)	0,250	0,310	0,370
Lactose (ml)	5,00	5,00	5,00

Dilute all standards to 100 ml in volumetric flasks. Sub-divide into 10 sachets (10 ml each) and freeze. Before each run, thaw a set of sachets (S1, S2 and S3). Dilute the standards (3 ml or 1 ml) to 100 ml for cells 1 and 2 respectively. Filter through 0,45μ into autosampler bottles. Prepare two vials for each of the three standards, plus (x+1) bottles of S2, the prime calibration standard, where x = no. of samples.

Samples

Weigh samples (1,0 g) in duplicate into 100 ml beakers. Accurately pipette 5 ml lactose solution into the beaker and then add about 50 ml of distilled water. Stir to dissolve. Transfer to a 100 ml volumetric flask and make to the mark. Dilute 3 ml or 1 ml of this solution to 100 ml for either cell 1 or cell 2 in a second volumetric flask. Filter through 0,45μ into autosampler bottles.

Running the samples

Run the 3 calibration standards first to establish the system's linearity. Replicate vials from each sample are then run, sandwiched between vials of S2. Average response factors from the S2 vials bracketing the samples are used to calculate sugar concentrations for each sample. This process is continued until all the samples have been chromatographed.

APPENDIX 3

Comparison of sugars in weekly molasses samples (50 samples from September 1991)

Mill	Fructose		Glucose		Sucrose		Mill	Fructose		Glucose		Sucrose	
	GC	IC	GC	IC	GC	IC		GC	IC	GC	IC	GC	IC
ML	9,1	9,2	8,2	8,0	31,7	31,7	ME	7,7	7,6	4,6	4,5	32,6	32,6
PG	6,0	6,1	3,5	3,6	30,0	30,0	GD	6,3	6,4	3,8	3,8	31,7	31,9
UF	4,7	4,8	2,6	2,6	33,0	33,2	GH	5,7	5,7	3,2	3,1	32,2	32,5
EN	4,6	4,7	3,3	3,2	33,5	33,6	NB	8,4	8,3	4,6	4,5	30,3	30,5
FX	6,2	6,3	4,4	4,3	30,3	30,4	UC	6,9	7,0	2,4	2,3	29,7	29,9
AK	5,7	5,9	3,8	3,8	31,0	31,0	IL	6,2	6,2	3,1	2,9	29,2	29,5
DL	6,5	6,7	3,7	3,7	31,5	31,5	SZ	7,3	7,2	4,1	4,1	29,3	29,5
MS	6,4	6,7	4,1	4,1	31,7	31,6	UK	7,1	7,2	3,9	3,8	30,7	31,2
ME	7,7	7,8	4,9	4,8	31,7	32,0	Check	11,1	11,0	9,9	9,7	26,1	26,4
GD	7,1	7,1	4,8	4,8	31,8	32,1	ML	7,4	7,6	5,9	5,8	31,8	32,0
GH	5,8	6,1	3,5	3,4	31,7	32,1	PG	6,3	6,4	3,8	3,8	29,6	29,6
NB	8,6	8,7	4,7	4,6	29,9	30,2	UF	5,0	5,1	3,1	3,0	33,4	33,7
UC	7,1	7,2	2,4	2,3	29,2	29,5	FX	6,2	6,2	4,2	4,1	30,4	30,8
IL	6,5	6,5	3,1	3,0	29,7	30,0	AK	5,3	5,4	3,3	3,2	31,8	31,9
SZ	7,7	7,8	4,6	4,5	29,2	29,6	DL	5,6	5,6	3,2	3,1	31,9	32,4
UK	7,2	7,2	4,1	4,0	31,0	31,3	MS	5,5	5,5	3,3	3,3	31,4	32,0
Check	4,0	4,1	1,9	1,9	36,5	36,3	ME	7,2	7,2	4,2	4,1	32,7	33,0
ML	8,2	8,3	7,0	6,9	32,2	32,3	GD	6,7	6,9	4,3	4,3	31,5	31,6
PG	6,2	6,3	3,8	3,7	29,5	29,6	GH	5,0	5,1	2,8	2,7	31,8	32,1
UF	4,6	4,6	2,5	2,4	33,9	34,0	NB	8,3	8,4	4,3	4,2	29,8	30,3
EN	4,3	4,3	2,9	2,8	32,3	32,4	UC	7,2	7,3	2,2	2,1	29,9	30,2
FX	6,3	6,4	4,3	4,2	30,1	30,5	IL	6,0	6,0	3,0	2,8	29,6	29,9
AK	5,7	5,8	3,6	3,6	31,6	31,7	SZ	5,9	6,5	3,2	3,5	29,5	29,7
DL	6,0	6,1	3,4	3,2	31,4	31,2	UK	6,6	6,9	3,6	3,6	31,0	31,4
MS	6,3	6,4	3,9	3,8	31,1	31,4	Check	7,6	7,7	5,5	5,4	31,5	31,4