

HPLC OF SUGARS THE ANALYTICAL TECHNIQUE FOR THE 1990's?

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Abstract

Various factory products have been analysed for sucrose, glucose and fructose by high performance liquid chromatography (HPLC). Comparisons between HPLC and gas chromatography (GC) have continued throughout the 1989/90 season for samples ranging from Direct Analysis of Cane (DAC) extracts to final molasses. Agreement has generally been very good. Analysis of trace sugars in condensates compared well with the classical phenol/sulphuric acid method. Comparison of the monosaccharide content of export sugars with reducing sugars by the Luff-Schoorl method was also good. In addition the technique was used to determine fermentation yields in a sugar cane distillery where glycerol and ethanol were also measured. The strengths and weaknesses of HPLC (including costs) are compared with those of direct pol and GC.

Introduction

Over the past five years progress in high performance liquid chromatography (HPLC) technology has been rapid. More efficient columns with relatively long working lives are now available. Microprocessor-controlled autosamplers and pumps can deliver extremely precise aliquots and flowrates. Modern refractive index detectors are temperature-controlled and have greater linearity and sensitivities. The time is therefore opportune to re-assess the use of HPLC in the SA sugar industry. The local sugar industry has used gas chromatography (GC) for cane payment and factory control since 1982 (Schaffler and Morel du Boil⁸). HPLC should have a number of advantages over GC and the possibility of HPLC taking over from GC therefore arises. Can these tasks be carried out by HPLC with the same degree of precision and accuracy as GC? Pol is traditionally used in mill laboratories for routine factory control and has the advantage of being precise and relatively cheap. However its accuracy decreases as purities decrease. Even in relatively high purity cane juices, detailed studies (Schaffler and Morel du Boil⁸) have shown that pol nearly always underestimates sucrose and that this bias is mainly dependent on invert levels. Should HPLC replace pol analysis in mill laboratories? Does the increased accuracy warrant the additional expense? These are some of the questions this paper will attempt to address.

Method and Materials

The equipment used for this study consisted of a Spectra-Physics (SP) IsoChrom pump and SP8875 autosampler, an Erma ERC-7512 refractive index detector, a Hewlett-Packard HP 3396A integrator and a column heater with controller manufactured in the Sugar Milling Research Institute (SMRI) workshop. Sample preparation involved a simple gravimetric dilution and filtration through a 0,45 μ membrane filter. For sodium-based columns, a temperature of 65°C was used, while 75°C was used for calcium ion columns. The calibration procedure was similar to that used in the GC analysis of sugars (Schaffler and Day-Lewis⁷). The main differences were the use of the external standard technique

and peak heights rather than peak areas. Three standards were chromatographed in duplicate at the beginning of each run to check linearity. Averaged response factors (RF) from the middle standard were used to calculate the concentrations of the sugars in the samples. A standard was injected after every six sample injections to allow for system drift. If the RSD's (relative standard deviations) of the initial and the most recent RFs exceeded 1%, the new RFs were used. Single injections of the three standards at the end of the run were used to determine recoveries of each sugar. Three columns were used during this study. Details are listed in Table 1.

Table 1
HPLC columns and conditions

Manufacturer	Column Type	Eluant	Column Temp. °C	Column Flow ml/min
Bio-Rad	HPX-87N	0,015M Na ₂ SO ₄	65	0,6
Shodex	S-801/S	0,01M Na ₂ SO ₄	65	0,6
Waters	Sugar-Pak 1	50 ppm CaEDTA	75	0,5

Before use mobile phases were filtered through 0,45 μ membranes and degassed by boiling for 5 minutes. The solvents were maintained at 75-85°C on a magnetic-stirrer/hot-plate to minimise carbon dioxide absorption and bacterial growth. Eluant was recycled through the column at 0,2 ml/min when not in use.

Results and Discussion

Optimisation

Column temperature can play an important part in the separation. Ivin⁴ showed that by varying column temperature interferant peaks could be moved relative to the major peak. To avoid on-column inversion, temperatures should be as low as possible. For sodium-based columns a temperature of 65°C was used, while 75°C appeared to be optimum for the calcium columns. During ruggedness testing, peak area measurements resulted in overestimation of sugars. For this reason peak heights were preferred as this mode is less susceptible to interferences from overlapping peaks.

Various factory products from DAC extracts through to final molasses were analysed.

DAC

DAC extracts from two cane deterioration tests were analysed by GC, HPLC and pol. A Bio-Rad HPX-87N was used and DAC samples were diluted 1 to 10 before injection. Estimates for sucrose by pol, GC and HPLC are shown in Figure 1. The differences between the three procedures are highlighted in Figure 2. Agreement between GC and HPLC was excellent, but pol/GC and pol/HPLC differences were highly significant and were due to the formation of invert during deterioration (Figure 3)

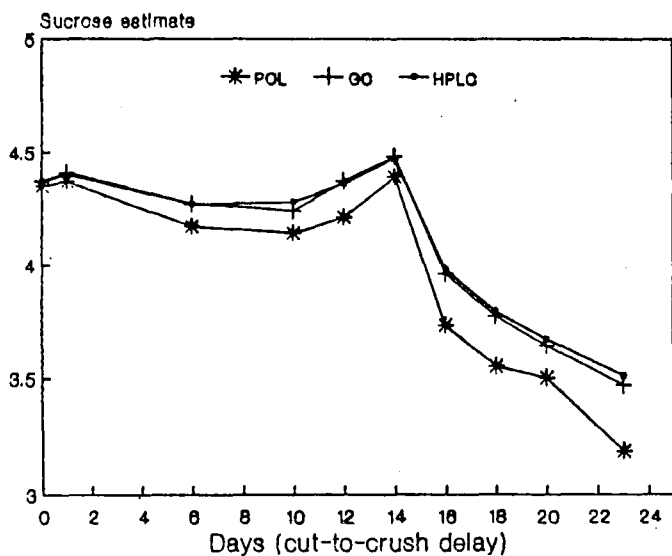


FIGURE 1 Comparison of HPLC with GC and pol data on DAC samples from deteriorated cane.

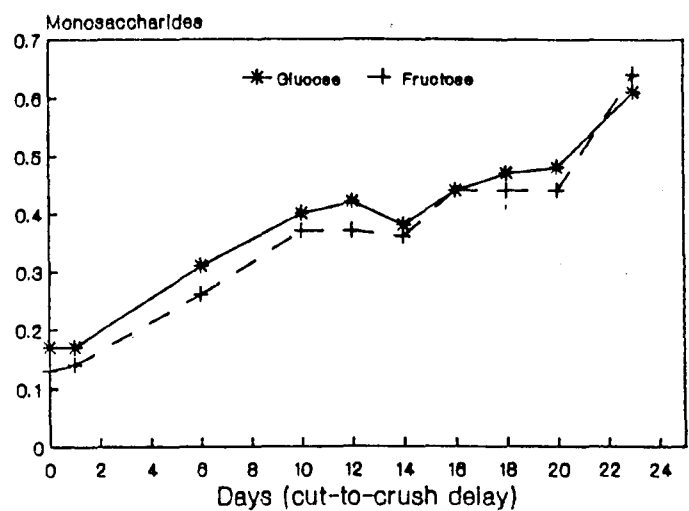


FIGURE 3 Monosaccharides in DAC samples by HPLC, effect of deterioration.

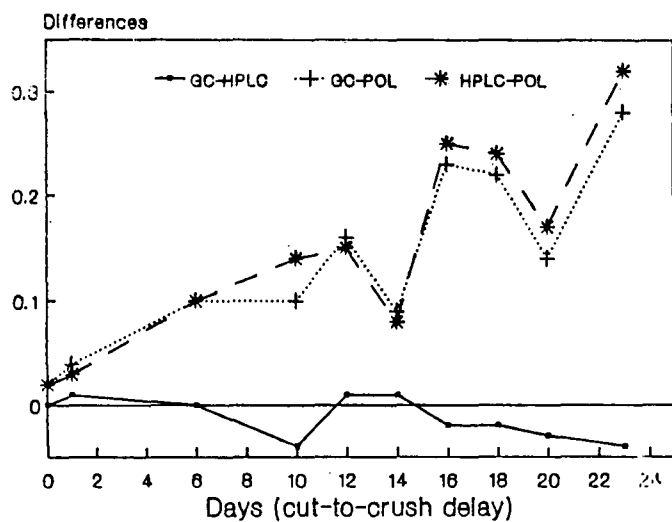


FIGURE 2 Sucrose in DAC samples. Comparison of HPLC, GC and pol differences.

Mixed Juice, Clear Juice and Syrup

Weekly samples of MJ, CJ and syrup, covering a period of 19 weeks, were obtained from PG sugar mill and analysed on two different columns. The average data obtained are compared with SICB GC data in Table 2.

Results from the two columns indicated that:

- (a) For MJ and CJ on the Shodex column, HPLC yielded virtually identical results to GC for all three sugars. This is indeed good news as this means that sucrose input to a factory could be determined by either technique with comparable results.

- (b) For MJ and CJ, the SugarPak column produced excellent results for the two monosaccharides but high results for sucrose.

- (c) Anomalous results were obtained for sucrose in syrup. The Shodex column produced results that were on average 0,6 units lower than the GC mean data whilst the SugarPak column produced results that were higher than GC by the same amount. Syrup is for all practical purposes a concentrated juice. The only analytical difference for HPLC was the dilution factor (50 times for syrup and 10 times for juice). The excellent agreement between GC and HPLC for juices using the Shodex column is therefore offset by the poor reproducibility for sucrose in syrup. Possible systematic bias in the HPLC or GC methods for syrup will be addressed during the 1990/91 season.

Final Molasses

Final molasses, being a low purity product is the most difficult stream to analyse reliably on a long-term basis. For this reason an interlaboratory chromatographic study involving four GC and two HPLC laboratories was conducted in 1989 (Day-Lewis²). Eleven monthly molasses samples were analysed. Both HPLC laboratories diluted samples a hundredfold with sugars being separated on Shodex columns. The precision of analytical techniques is normally monitored using the repeatability (r) and reproducibility (R) concepts (Anon¹, Youden and Steiner¹⁰). Both parameters have been defined elsewhere (Mellet *et al.*⁶). Statistical analysis showed that the within laboratory variance was exceptionally low for all six laboratories and for all three sugars (r = 0,1 to 0,2 units). No significant differences were noted between the two techniques for any of the sugars. The 2-way Anova data are presented in Table 3.

Table 2 Comparison of HPLC with GC data for MJ, CJ and syrup

Column	n	Sample	Sucrose			Glucose			Fructose		
			GC	HPLC	Diff.	GC	HPLC	Diff.	GC	HPLC	Diff.
Shodex	12	MJ	10,47	10,47	0	0,25	0,24	+0,01	0,24	0,24	0
		CJ	10,68	10,72	-0,04	0,25	0,24	+0,01	0,23	0,24	-0,01
		Syrup	52,70	52,12	+0,58	1,34	1,27	+0,07	1,20	1,23	-0,03
SugarPak	7	MJ	10,41	10,52	-0,11	0,21	0,21	0	0,23	0,24	-0,01
		CJ	10,97	11,19	-0,22	0,22	0,21	+0,01	0,23	0,23	0
		Syrup	51,72	52,32	-0,60	1,18	1,13	+0,05	1,21	1,19	+0,02

Table 3

ANOVA data from an interlaboratory study on 11 molasses samples (Sugars expressed as % on mass)

Laboratory	Fructose		
	Sucrose	Glucose	Fructose
SMRI GC	28,4	6,3	8,1
STD GC	28,5	6,5	8,1
SICB GC	28,5	6,4	8,2
NCP GC	28,1	6,6	8,2
SMRI LC	28,5	6,3	8,3
NCP LC	28,5	6,4	8,1
Mean	28,4	6,4	8,2
F-value	2,9	2,8	0,6
Repeatability	0,2	0,2	0,1
Reproducibility	0,9	0,6	0,9

Reproducibility (R, i.e. interlaboratory precision for both GC and HPLC results) was calculated and found to be 0,9; 0,6 and 0,9 units for sucrose, glucose and fructose respectively. These precision estimates may appear to be high. However the sugar industry has been influenced by the very close reproducibility that can be found in polarisation measurements, thus setting unrealistically high standards for other measurements.

Precision estimates by Godshall³, Mellet *et al.*⁶ and ICUMSA produced R-values of 0,8; 0,4 to 1,0 and 1,0 respectively. All these were considered to be acceptable. The HPLC results summarised in Table 3 are therefore also considered to be in good agreement with the GC results. It must also be remembered that repeatability and reproducibility are probability estimates and may seem inflated when compared to more familiar parameters (e.g. standard deviation or coefficient of variation).

An ICUMSA HPLC study is currently being evaluated, involving six samples and analysis by fourteen laboratories. Preliminary HPLC data from two S A laboratories are compared with GC mean data from three laboratories in Table 4.

Table 4
Comparison of HPLC with GC data : ICUMSA 1990

Method	Samples					
	A	B	C	D	E	F
GC	30,5	29,4	30,1	32,3	35,4	33,4
HPLC	30,8	29,6	30,4	32,3	35,5	33,2
			R-GC = 0,7			
			R-HPLC = 0,9			
			R-overall = 0,8			

Glucose

Method	Samples					
	A	B	C	D	E	F
GC	3,6	3,7	4,7	2,5	3,2	3,6
HPLC	3,6	3,7	4,7	2,6	3,2	3,5

R-GC = 0,3
R-HPLC = 1,3
R-overall = 0,1

Fructose

Method	Samples					
	A	B	C	D	E	F
GC	6,1	7,1	7,8	5,5	5,2	4,3
HPLC	6,2	7,3	7,9	5,5	5,2	4,3
			R-GC = 0,5			
			R-HPLC = 1,6			
			R-overall = 0,4			

The HPLC results were obtained from one SugarPak and two different Shodex columns. The agreement between GC and HPLC for sucrose was excellent (R = 0,8). In addition laboratory-to-laboratory scatter for both methods was very similar (R = 0,7 and 0,9).

For the monosaccharides, HPLC and GC means compared well; however the HPLC inter-laboratory scatter ($R_{HPLC} = 1,3$ to 1,6) was almost three times greater than for GC. No reason could be found for the greater spread. During 1990 parallel GC/HPLC analyses will continue and greater emphasis will be placed on column resolution and calibration drift. Despite this slight drawback, the current HPLC method is considered to produce results similar to those from GC and adequate for routine factory control.

Distillery Liquors

The great advantage of HPLC analysis for distillery samples is that sucrose, glucose, fructose, glycerol and ethanol can be determined in a single run (Figure 4).

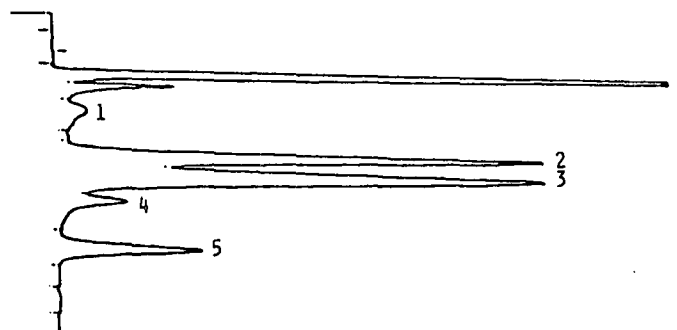


FIGURE 4 Chromatogram of a distillery liquor. Peak identification is 1 sucrose, 2 glucose, 3 fructose, 4 glycerol and 5 ethanol.

Due to the high solids contents, a pre-filtration through a slow paper (e.g. Whatman No. 6) prior to membrane filtration is required. As an example ethanol results by both HPLC and GC for distillery slops are presented in Table 5.

Table 5
Comparison of HPLC with GC data for ethanol % in distillery slops

Sample	GC	HPLC
1	0,24	0,25
2	0,24	0,27
3	0,33	0,32
4	0,23	0,23
5	0,20	0,20

Condensates

Traditionally the refractive index (RI) detector has been relatively insensitive. However the Erma RI has built-in temperature control, giving reduced noise and increased sen-

sensitivity. The HPLC system was tested on factory condensates and compared with results from the traditional phenol/sulphuric acid analysis. Carbohydrates can also be determined using an anion exchange column and pulsed amperometric detection (PAD) (Tsang *et al.*⁹, Lee and Bunker⁵). This relatively new technique offers even greater sensitivity and selectivity than cation exchange/RI HPLC. Comparisons between the three techniques are presented in Table 6. For simplicity, the individual sugars, determined by HPLC, are reported as total sugars.

Table 6

Comparison of HPLC with PAD and phenol/H₂SO₄ for sugar traces (ppm) in condensates

Sample	Phenol/H ₂ SO ₄	HPLC PAD	HPLC RI
V1	21	18	17
V2	2	2	1
V3	1	1	2
Xh	0	0	0
E2	0	0	0
E4	30	28	29
E5	26	26	29
Xh	0	0	0
IL-K	0	0	0
-V1	2	2	1
-V2	0	2	2
-V3+4+5	2	5	0
SZ-V4	37	26	26
-Xh	12	0	1
-Feed	3	0	0

Agreement between the two HPLC procedures was excellent, and both agreed well with the phenol/sulphuric acid method.

Raw Sugars

Export (VHP, SHP and ship) sugars were analysed at 1% dilution for sucrose and 10% dilution for monosaccharides. The sucrose results were in general 0,3 units higher than pol. Although the RSD of duplicates was less than 0,4 in all cases, this precision cannot compare with that of pol. It is doubtful whether precision in HPLC can be significantly improved to make it a viable alternative to pol for sugars.

The fructose and glucose results obtained by HPLC were compared with reducing sugars as determined by the Luff-Schoorl method. The invert levels in VHP sugars were too low for detection using RI-based HPLC analysis. Results for SHP and shipment sugars are detailed in Table 7. HPLC values were approximately 0,1 units higher than the chemical procedure.

Table 7

Comparison of HPLC with Luff-Schoorl for invert in SHP and coated sugars

Sample	Fructose	Glucose	Fructose + Glucose	Luff-Schoorl	(Fructose + Glucose) - Luff-Schoorl
SHP 1	0,15	0,15	0,30	0,19	+0,11
SHP 2	0,20	0,20	0,40	0,25	+0,15
SHP 3	0,10	0,11	0,21	0,12	+0,09
LP	0,57	0,60	1,17	1,10	+0,07
HP	0,31	0,31	0,62	0,55	+0,07
SW	0,20	0,22	0,42	0,43	-0,01
Average			0,52	0,44	+0,08

Column Comparisons

Both the Bio-Rad HPX-87N and the Shodex S-801/S columns have a sodium counter-ion as opposed to the SugarPak where calcium is used. Separation between glucose and fruc-

tose was superior with the calcium column, but these columns are reputed to be less robust and to have a shorter life. Our experience with SugarPak columns indicates a column life of 700-800 injections. The Bio-Rad column, which is still in use, has withstood 1 400 injections. The Shodex column was still working extremely well when it was removed after 840 injections. This column in other laboratories has been known to withstand ±10 000 injections (Cornish L, personal communication). Analysis-time was similar for all columns (approximately 10 to 15 minutes). Separation of inorganic compounds and higher saccharides from sucrose appeared to be adequate on all columns.

Costs

Comparisons of capital and running costs for GC, HPLC and pol are shown in Table 8.

Table 8

Comparison of capital and running costs

	HPLC	GC	Pol
Instrumentation (in thousands)	R91-R96	R79-R99	R88-R96
Column	R3 870-R4 440	R1 300-R1 700	N/A
Cost/sample	R4	R11	R1
Cost/sample including column	R7	R11	N/A

Notes: All prices are inclusive of 13% General Sales Tax
An exchange rate of US \$ 1 = R2,70 has been assumed.
Running costs do not include labour.

Table 9

Advantages and disadvantages of HPLC, GC and Pol

	HPLC	GC	Pol
Sample preparation	simple	involved	simple
Ease of analysis	moderate	moderate	easy
Sample toxicity	none	high	high
Precision	high	high	highest
Calibration	yes	yes	no
Accuracy	high	highest	can be low
Sensitivity	medium	high	low
Fructose + glucose	yes	yes	no
Trisaccharides	yes	yes	no
Cost	high	highest	low

Instrument prices include an autosampler, column and integrator. Cost per sample has been calculated assuming a run of 16 samples in duplicate with one set of standards, but does not include labour. Capital costs are similar for all

three techniques. Column life for GC capillary columns is exceptionally good with an estimated life of 5 000 injections. Column life for HPLC is still uncertain, but 1 000 to 2 500 is a likely range. These estimates have been used to calculate the cost per sample including column. Even with the greater column costs of HPLC, the total cost/sample is less than that of GC. Manpower costs would also be less because of the simpler sample preparation.

The relative advantages and disadvantages of the three techniques are shown in Table 9.

It is obvious from Table 9 that the polarimeter will still be required for routine analysis, especially of raw sugars. Although HPLC is still relatively costly, it could have a definite future in the sugar industry for accurate estimates of sucrose in low-purity products. Its success will depend on reducing running costs as well as adequate training of factory laboratory staff. Effective use of the additional information will also determine HPLC's success. Some obvious examples are monitoring of inversion and Maillard reactions and possibly boiler-water contamination. When legislation limiting the use of lead acetate is introduced, HPLC could be a viable alternative for pol. GC is currently used for cane payment and factory performance measurements. HPLC offers faster and simpler sample preparation. However interlaboratory scatter is still greater than with GC, and this needs further investigation. It is envisaged that HPLC could be a viable alternative to GC in the next 1 to 2 years.

Conclusions

Comparisons of HPLC data with GC for analysis of juices and final molasses are extremely favourable for all three sugars. The relative simplicity and lower cost of HPLC suggest that there is a definite possibility that HPLC could replace GC for cane payment and molasses analyses. However, inter-laboratory scatter is greater than that for GC laboratories. Before a final decision is made, it would be advisable to run HPLC in tandem with GC on all mixed juice and final molasses samples for at least one season.

HPLC has been shown to produce useful and accurate information on DAC extract quality, and is the ideal analytical technique for distillery monitoring. With a high sensitivity RI detector it is also sensitive enough to monitor sugar traces in condensates.

Analysis of raw sugars by HPLC for sucrose does not appear to be viable. However, analysis of SHP and coated sugars for fructose and glucose gives reasonable agreement with reducing sugars as determined by Luff-Schoorl titration.

For a mill laboratory the great advantages of HPLC over GC are simplicity and speed. Sample preparation involves only gravimetric dilution and filtration. Thus a result could be available within half an hour of the sample being taken. The advantage over pol is greater accuracy, especially for low purity products, with the bonus of monosaccharide concentrations being determined in the same run.

The capital cost of the instrumentation for the three techniques is similar. Operating costs for HPLC are approximately half those of GC. Pol is clearly the most economical of the three procedures.

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