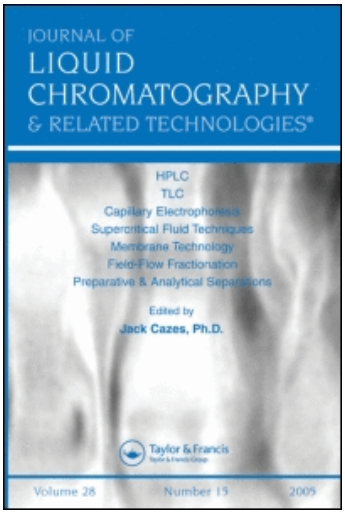

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Duarte-coelho, A. C. , Dumoulin, E. D. and Guerain, J. T.(1985) 'High-Performance Liquid Chromato-Graphic Determination of Sucrose, Glucose, Fructose in Complex Products of Distilleries', Journal of Liquid Chromatography & Related Technologies, 8: 1, 59 — 73
To link to this Article: DOI: 10.1080/01483918508067063
URL: http://dx.doi.org/10.1080/01483918508067063

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(1), 59-73 (1985)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF SUCROSE, GLUCOSE, FRUCTOSE IN COMPLEX PRODUCTS OF DISTILLERIES

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ABSTRACT

Sucrose, glucose, fructose, present in complex products, juices, wines, molasses, vinasses, were determined by high performance liquid chromatography (HPLC). The sugars were separated on Waters Sugar Pak column in the Ca^{++} form, using water with calcium acetate (20 mg/l) as the mobile phase, and a flow rate of 0,5 ml/min. The column was heated to 90°C and the sugars were determined using refractive index.

Reproducible sample preparations were proposed, according to the product. Preparation and analysis required 35 minutes. Results were compared with other analytical methods.

 0148-3919/85/0801-0059$3.50/0

INTRODUCTION

In the last years, ethanol became an important energetic product and it is justified to optimize its production.

Alcoholic fermentation of sugared juices produced from beets and graps or molasses, gives a wine containing 7 to 9 % v/v ethanol, which will be distillated.

The optimal production of a distillery is obtained by monitoring processes and by establishing balances, to perform a good gestion of material and energy.

Initial saccharide content gives the potential alcoholic production. Saccharide, acid, alcohol contents in the final products (principal or by-product) inform about the efficiency of the processes. During the fermentation, analysis of intermediate products can inform about sugars evolution, possible anomalies and provide a help to carry out fermentation in the most economical conditions, before distillation.

Most analytical methods need a long sample preparation. Usual methods are : polarimetry for saccharose ; chemical oxydation for total reducing sugars, silylation, obtention (24 h) of volatil derivates of the different sugars, analyzed by gaz chromatography ; enzymatic oxydation of sugars and alcohols.

Concurrently to these methods, we choose to study high performance liquid chromatography. This technique permits an efficient separation of sugars, sucrose, glucose, fructose and alcohols, glycerol, ethanol.

Many works use HPLC for sugars determination in food products (1, 2, 3), fruits (4), cereals (5), dairy products (6, 7) and in wines (8, 9), beers and worts in fermentation (10). Generally they used Aminex or Bondapak carbohydrate columns, and as mobile phases, water or water + calcium salt for Aminex, and water + acetonitrile for Bondapak. Morawski (8) proposes HPLC as a tool to determine kinetics of transformation of glucose into ethanol, using a Sugar Pak columns and water as the mobile phase. Preparations of studied products samples always need 20 to 30 min, and 15 min for separation and analysis.

The purpose of this work was to develop a rapid automatised method of sample preparation, before the separation of sugars by HPLC, and their quantitative determination, in the different com-

plex products of distilleries. Results are compared with other analytical methods : total reducing sugars and silylation.

MATERIALS AND METHODS

Apparatus

High pressure liquid chromatograph : Waters Associates model Sugar-1 equipped with refractive index detector Waters R 401

HPLC column : Sugar-Pak-I (Waters Ass.), 30 cm x 4 mm id ; Ca form ; temperature 90°C

HPLC injection valve : model Rheodyne (Waters Ass.) with 25 µl loop

Automatic injector : Waters-590

Recorder : model Y-T "Omni-Scribe"

Syringe : 1 ml capacity ; B-D, Yale Tuberculin, N°2027 (1YTL)

Reagents

Regenerating solution : deionized water (Amberlite MB-3 analytic Prolabo), filtered under vacuum through 0,4 µm Nuclepore membrane ; addition of 200 mg/l calcium acetate and 100 mg/l Thiomersal (preservative) ; keeped at 65°C and continuously degassed by use of magnetic stirrer.

Mobile phase : regenerating solution diluted with deionized and filtered water, to obtain a 200 mg/l calcium acetate solution ; flow rate 0,5 ml/min ; pressure 60 bar.

Sugars and alcohols standards : sucrose, fructose, glucose, ethanol absolute (reagent grade, Merck), glycerol (quality R.P., Prolabo).

Mixed Standard Solutions

Using deionized filtered water :

I - 0,5 % w/v (or 0,5 g/100 ml) of each sugar, sucrose, glucose, fructose and glycerol ; and 0,5 % v/v (or 0,5 ml/100 ml) of ethanol.

II - Dilutions of I in water : 0,25 % ; 0,1 % ; 0,05 % ; 0,025 % ; 0,01 %.

III - 13 % sucrose ; 1 % glucose and fructose (same proportions as a diffusion juice).
IV - 9 % v/v ethanol ; 1 % w/v glycerol ; 0,02 % w/v glucose and fructose (same proportion as a wine after fermentation of the diffusion juice).
Prepared daily to ensure stability of standard solutions.

Complex Products of Distillery

Beet juice (or diffusion juice), beet wine (after fermentation), molass, vinass, piquette : these products were congelated during production time 1983-1984.

Classical Preparation for Complex Products

To 100 ml sample, add 10 ml lead subacetate (density 1,33 kg/l) ; decant, then add $CaCO_3$ (2 g) to precipitate lead acetate excess ; filter on Durieux n°3 paper. Then pass on ion exchanger resin (MB-3 Anal. Prolabo) and filter through Millipore membrane (Milex réf. 185996 ; 0,4 µm).

Operating Conditions HPLC

Rinse the syringe with sample, 3 times. Then, rinse the loop of injection (20 µl) 4 or 5 times with sample (1 ml). This is particularly necessary between two different injections of samples containing respectively 1 and 0,01 % sugar.

Inject 20 µl of sample into chromatograph. Measure retention times and peak heights, and compare with those obtained for standard solutions.

Calculate individual sugar (or alcohol) content in sample as follow :

$$\text{sugar \% w/v} = \frac{H}{H'} \cdot x \cdot 100 \qquad \text{(or alcohol \% v/v)}$$

where H and H' = the peak heights of sugar in sample and standard respectively

x = % w/v sugar in the standard solution (or % v/v alcohol).

Daily or after passing 0,5 to 1 l of mobile phase, regenerate column with regenerating solution flow 0,5 ml/min during 5 h.

RESULTS AND DISCUSSION

Separation of Saccharides and Alcohols from Standard Solutions

With the mixed standard solution I containing 0,5 % w/v sugars and glycerol, and 0,5 % v/v ethanol, we obtained the separation shown in Fig 1.

Retention times (min) for sucrose, glucose, fructose, glycerol, and ethanol were 7,3 ± 0,2 ; 9,2 ± 0,2 ; 10,3 ± 0,3 ; 11,8 ± 0,2 ; 12,7 ± 0,3 respectively. Peak heights measured for 10 injections give a relative error inferior to 1,5 %.

For several dilutions of solution I, we obtained, by plotting peak heights versus component content (Fig 2), a linear variation of height in the range 0,01 to 0,5 % w/v for sugars and 0,01 to 0,5 % v/v for alcohols. 0,01 % was the minimum level of each sugar and alcohol that could be quantified with the experimental conditions.

Separation of Saccharides and Alcohols from Complex Products

After submitting samples to "classical preparation", we injected prepared sample solution in the chromatograph. For each different product, we prepared a standard sample with similar sugar and alcohol contents, that gave peak response ± 10 % of sample peak response. Standard solution III was used for diffusion juice and IV for beet or molass wines, and for molass vinass.

Beet Wine

It was necessary to use different sensibilities to detect sugars, glucose and fructose (0,02 % w/v) and ethanol (9 % v/v).

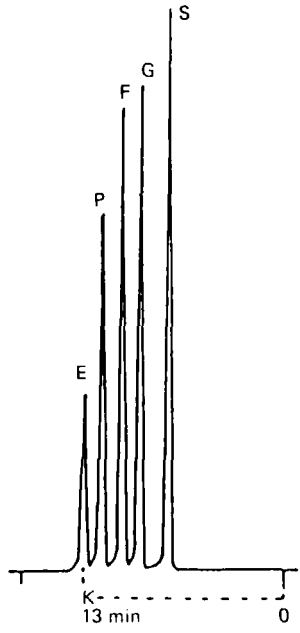

FIGURE 1. Separation of sucrose (S), glucose (G), fructose (F), glycerol (P), ethanol (E), from standard solutions (5 mg/ml S, G, F, P ; 5 ml/ml E) on Sugar Pak, Ca^{++} form.
Temperature 90°C ; pressure 60 bar ; mobile phase : water + 20 mg/l calcium acetate ; flow rate 0,5 ml/min ; detector Waters refractive index (16X).

Retention times (min) for glucose, fructose, glycerol and ethanol were 8,7 ; 10,8 ; 12,7 ; 13,3 respectively (6 min for raffinose).

For sugars, we were at the minimum detection limit values. With the sensibility 32X, we obtained variable peak heights for ethanol (3 injections : 245, 235, 185 mm) but constant for glycerol (60 $\pm$ 1 mm).

Ethanol is a volatile component, so it would be better to use an automatic injection for quantitative determinations.

Diffusion Juice

Stable retention times (min) for sucrose, glucose, fructose (7,3 ; 8,6 and 10,3 respectively) were observed. Peak heights mea-

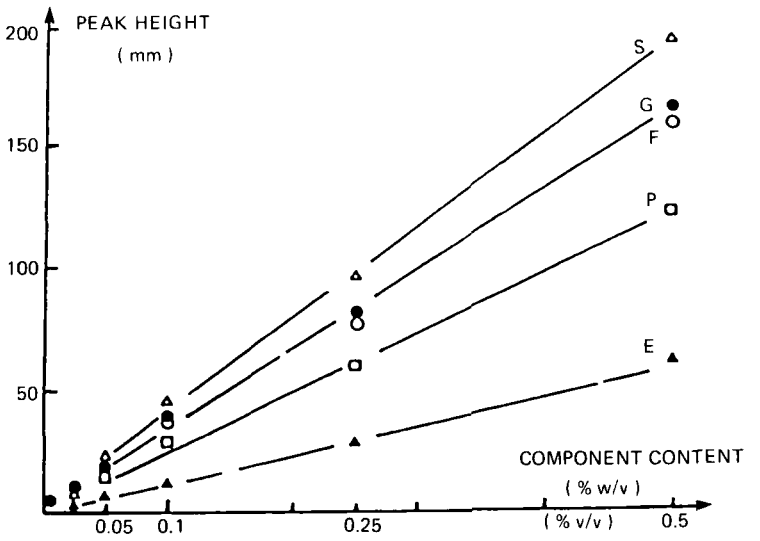

FIGURE 2. Peak height for dilutions of standard solution (5mg/ml S, G, F, F ; 5 ml/ml E).

sured for 5 injections showed a relative variation inferior to 1 %. With standard solution III, diluted to obtain 6,5 % w/v sucrose, and sensibility 32X, we calculated the sample contents : sucrose 6,7 %, glucose 1,8 %, fructose 1,5 %, and glycerol 0,1 %. Probably sucrose was partially consumed during storage ; glycerol was contained in vinass, used in the process as a diffusion diluent.

Comparison with other Methods

Quantitative results were compared with those obtained for total reducing sugars by chemical determination, and for each sugar by silylation (TMS derivatives and GLC).

The different chromatograms obtained for the different products, beet wine, piquettes, molass wine and molass vinass are shown on Fig. 3 and the calculated sugar contents are presented in Table 1.

TABLE 1

Comparison of Sugars Contents (% weight/volume)
determined by Different Methods
(G : glucose ; F : fructose ; ST : G+F = total sugars)
(piquette : by-product of grape - wine preparation)

PRODUCTS	HPLC (16X)			SILYLATION			TOTAL REDUCING
	G	F	ST	G	F	ST	"SUGARS"
BEET WINE	0,02	0,02	0,04	0,06	0,05	0,11	0,18
"PIQUETTES" 1	0,42	0,42	0,84	0,41	0,34	0,75	1,4
"PIQUETTES" 2	0,11	0,33	0,44	0,11	0,34	0,45	0,72
"PIQUETTES" 3	0,11	0,27	0,38	0,06	0,07	0,13	0,56
MOLASS WINE	0,15	0,28	0,43	0,03	0,34	0,37	0,61
MOLASS VINASS	0,17	0,21	0,38	0,04	0,07	0,11	1,03

Each HPLC value was a mean value obtained from two "classical" sample preparations, each prepared sample being injected 5 times.

The relative variation of different sugar content determinations was 10 %.

Chemical method gave results by excess : not only the sugars, but all the reducing substances were concerned.

Results of silylation and HPLC methods were in good agreement if we considered the very low level of sugar content determination.

For the wines, we also obtained peaks corresponding to ethanol and glycerol, but the heights were not constant for ethanol.

These results showed us that it was possible to separate and determine quantitatively sugars in complex products. But the "classical sample preparation" lasted one hour, which is a short time compared with the silylation method, but still too long to lead to a rapid automatic method.

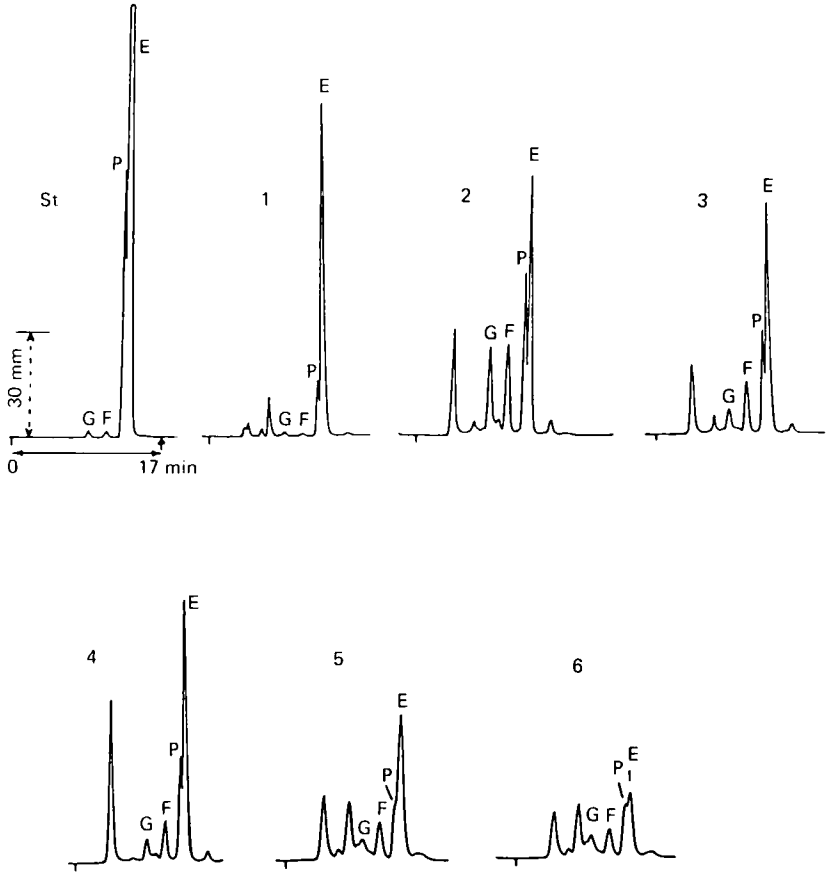

FIGURE 3. Chromatograms of complex products : classical preparation. HPLC conditions of Fig. 1 (16X).
St : standard (E : 9 % v/v ; P : 1 % w/v ; G, F : 0,02 % w/v) ; 1 : beet wine ; 2, 3, 4 : piquettes ; 5 : molass wine ; 6 : molass vinass

Modified Sample Preparation

We proposed two methods, according to the sugar content :

A - for the products with a sugar content inferior to 0,5 % w/v, apply the classical preparation untill the addition of $CaCO_3$; then add 60 g of resin Amberlite, shake 5 min, centrifuge 10 min and filter on Millipore membrane (Millex 0,4 μm).

B - for the products with a sugar content superior to 0,5 % w/v, dilute a 10 ml sample to 100 ml, then add 10 g resin (Amberlite MB-3) shake 5 min, centrifuge 10 min and filter supernate on Millipore membrane (Millex 0,4 μm).

Sample preparation A was used for different products. Except for beet wine, the other products were diluted (x 10) before preparation (Fig. 4). Standard solutions and samples were submitted to the same preparation and all of them were injected several times.

Results are reported Table 2. The relative variation of peak height measure was 10 %, for all the injections.

TABLE 2

Sample Preparation A used for different Products

PRODUCTS	Numbers of injections	HPLC S	G	F	Tot.	TOTAL REDUCING "SUGARS"
BEET WINE (16X)	8	-	0,05	0,08	0,13	0,18
PIQUETTE (4X)	3	-	0,31	0,31	0,62	1,38
MOLASS WINE (8X)	2	-	0,65	0,61	1,26	0,61
DIFFUSION JUICE (8X)	8	11,7	3,1	2,3	17,7*	15,01

* sucrose calculated as glucose equivalent

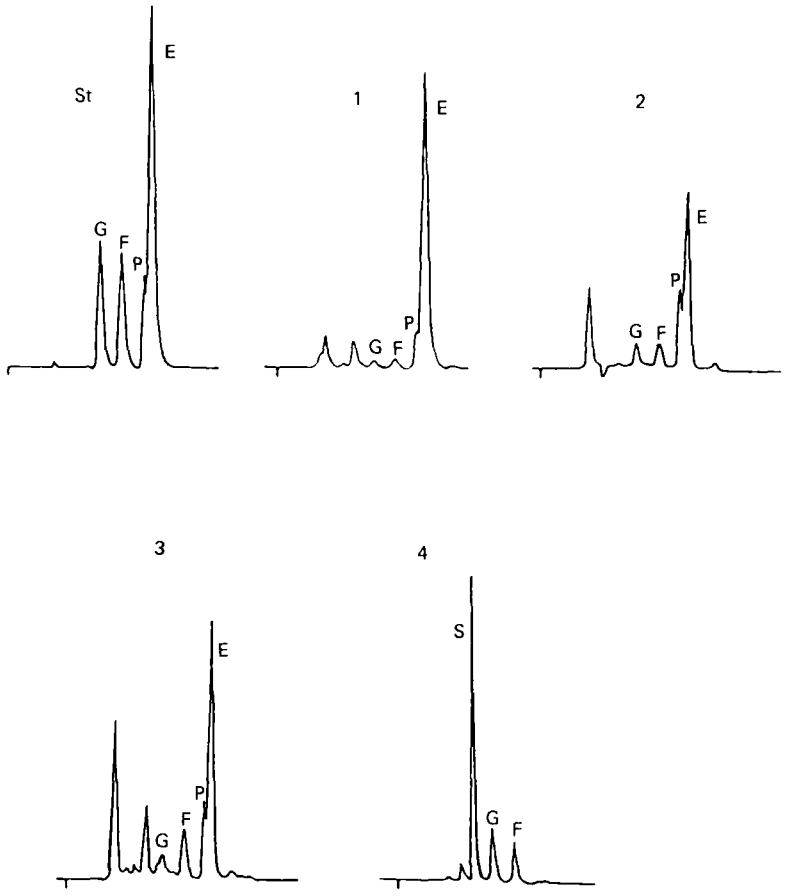

FIGURE 4. Chromatograms of complex products : modified preparation A. HPLC conditions of Fig. 1.
St : standard (4X) (E : 9 % v/v ; P, G, F : 1 % w/v) ; 1 : beet wine (4X) ; 2 : piquette (8X) ; 3 : molass wine (8X) ; 4 : diffusion juice (8X).

During sample preparation A, we observed, by comparing filtrations through membranes Millex Millipore (0,4 μm)and Sep-Pak Waters (50 μm), that fructose seemed to be retained on Millex more than on Sep-Pak. 5 samples were filtered through each membrane, giving fructose contents in wine 0,05 % ($\pm$ 0,01) (Sep-Pak) ; but Millex filtrates were clearer.

Sample preparation B was used for five samples of the same piquette. Then each sample was injected 5 times. Standard solution was prepared in the same way and injected 5 times. Results are reported Table 3.

By Grubbs test, with a 95 % probability we could say there was no aberrant value for each 5 injections groupe.

Reproducibility was good for the preparation B, used for the very low sugars contents.

Each method, A or B can be realized in a time inferior to 20 minutes.

Added Sugars Recovering (preparation B)

The accuracy of the method was assessed on the basis of recovery of sugars added to the sample, prior to the preparation. Essays were performed both on standard solutions and complex products :

- in the range 0,05 % of each sugar fructose and glucose, after adding quantities in the same order of magnitude, we obtained 99,9 % recovery
- for glycerol and ethanol in the range 0,4 % w/v and 6,3 % v/v respectively, we obtained 98 and 97 % recovery. This method could be used for alcohol determination.

Study of the different Steps of the Preparations

With melass wine sample, a very complex product, still containing yeasts (2,5 %), we tried to explain the part played by each step of preparations. We obtained the following results :

TABLE 3

Results of 5 sample Preparations B for Piquette

Average of 5 injections

(G : Glucose ; F : fructose ; P : glycerol ; E : ethanol)

PRODUCTS	G % w/v	F % w/v	P % w/v	E % v/v
STANDARD	0,41	0,46	0,69	3,20
SAMPLES 1	0,52 ± 0,06	0,47 ± 0,04	0,96 ± 0,05	3,45 ± 0,6
2	0,53 ± 0,02	0,45 ± 0,02	0,90 ± 0,02	3,11 ± 0,4
3	0,51 ± 0,01	0,43 ± 0,01	0,86 ± 0,03	3,11 ± 0,3
4	0,50 ± 0,01	0,44 ± 0,02	0,87 ± 0,02	3,26 ± 0,5
5	0,48 ± 0,01	0,41 ± 0,01	0,85 ± 0,01	3,11 ± 0,4
MEAN VALUE	0,51	0,44	0,89	3,21

- the filtration of pure of diluted (1/10) melass wine through an organic membrane (Millex ref. 185 996 0,4 µm or Millex GS 0,22 µm) or a mineral membrane (0,2 µm) gave numerous and unseparated peaks
- the treatment of pure or diluted (1/10) melass wine with resin (Amberlite MB3) gave well-separated peaks of fructose and glucose. The resin seems to be the principal element of the preparation but some peaks must be eliminated to protect the chromatographic column
- the treatment of defecation with lead subacetate or lead acetate, eliminated head and tail peaks, but had a negative effect on fructose and glucose peaks (retention or peak appearance). On the other hand, the filtration on mineral membrane (40 Å) permitted to eliminate head and tail peaks, and to preserve fructose and glucose peaks. In this last case, it was not necessary to dilute the wine.

All these observations lead us to propose two possibilities of preparation :

- the use of the preparation B, without centrifugation
- an automated preparation where the product (without dilution) could be filtered through a mineral membrane (40 Å), passed on resin, then through a simple filter to avoid to carry resin parti-

cles towards the chromatographic column. In less than 5 minutes, the sample would be ready to be analyzed by HPLC (25 to 30 min).

CONCLUSION

HPLC could be an analytical instrument to monitor sugars evolution in distillery processes.

Specific preparations were tested for different complex products. Total time for one analysis was about 35 minutes. The preparation methods proposed in this paper, were reproducible, in good agreement with other classical methods, and could readily be automated.

AKNOWLEDGEMENTS

This study, part of a research program on distillery, was supported by D.I.A.A., Agricultur Ministery in France.

A.C. Duarte-Coelho, an ingenior from Bresil, received an help from the "Conselho National de Ensino e Pesquisa" of Bresil.

H.P.L.C. equipment was lended by Waters Associates in France.

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