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SHORT COMMUNICATION

FULL OPTIMIZATION AND VALIDATION OF AN HPLC METHOD FOR THE QUANTITATIVE ANALYSIS OF TOTAL SUGARS IN A SOFT DRINK

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ABSTRACT. Five HPLC methods were employed for the quantitative analysis of three natural sugars namely fructose, glucose and sucrose in soft drinks. HPLC-refractive index detector (RID)-AMINO proved to be the most suitable HPLC method to carry out the latter task. For the optimum separation and response of the natural sugars the best conditions employed were column oven temperature 30 °C, flow rate 0.1 mL/min, mobile phase ratio acetonitrile:water 75:25 and they were determined by studying all possible interactions among these three parameters. Full validation of HPLC-RID-AMINO was performed in terms of system suitability test, precision check, accuracy check and robustness.

KEY WORDS: Sugar, Soft drink, Experimental design, Validation, HPLC, System suitability

INTRODUCTION

Diabetic patients are advised to limit their sugar intake [1, 2] in compliance with the studies showing that simple sugars can cause higher postprandial glycemia than starch [3]. Hence, to avoid problems such as stimulating hyperglycemia, having recourse to insulin [4, 5], and causing possible cardiomyocyte dysfunction [6, 7] and/or enhanced loss of β-cells [8] diabetic patients have diets low in sugar.

In context of Mauritius, a population group of 25 years and over, 12.7% (i.e. 52,000 individuals) have diabetes and a further 17.5% (or 83,000 individuals) have impaired glucose tolerance, whereas in the population group of 45 years and over, 23% (or 42,000 individuals) have diabetes and a further 22% (or 40,000 individuals) have impaired glucose tolerance. Thus amongst Mauritian adults aged 45 years and over, approximately 1 in 2 people have diabetes, or have a high risk of developing it [9].

High performance liquid chromatography (HPLC) is the most adopted technique for the separation and quantification of individual natural sugars namely glucose, fructose and sucrose [10]. Non-structural carbohydrates may also be isolated and quantified using HPLC coupled with the relevant columns and detectors such as refractive index detector (RID), evaporative light scattering detector (ELSD) and pulsed amperometric detector (PAD) [10-12].

However, in the literature little work has been undertaken to determine the optimal conditions for the separation of these sugars in terms of flow rate, oven temperature and mobile phase when having recourse to HPLC. The three mentioned parameters are very essential in providing the best response, separation and resolution of these sugars through HPLC. The work presented in this paper focuses on how to come up with the best suited HPLC method with respect to system suitability (resolution and specificity), method performance (precision, accuracy and specificity), volume of sample used, organic wastes generated and cost and on how to determine its optimum conditions that would allow simultaneous separation, detection, identification and quantification of the three natural sugars present in a sample of a soft drink.

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Most of the soft drinks have more or less the same composition in terms of ingredients such as acidity regulator, additives and sugars, hence, a commercially available soft drink has been chosen as an analyte since it is widely consumed in Mauritius and so can be possibly linked to the high prevalence of diabetes in Mauritius due to its very high sugar content. In addition, it was important to devise such a method to quantify the amount of total sugars in soft drinks since the Government of Mauritius has imposed a tax of 3 cents for each gram of sugar present in these soft drinks [13].

EXPERIMENTAL

Reagents. Standards for fructose, glucose and sucrose of high purity (99.5%) were purchased from Merck which were used as calibration standards. Certified reference standards for fructose, glucose and sucrose were obtained from Sigma Aldrich, Germany, were used to prepare quality control check (QCCheck). A food analysis performance assessment scheme (FAPAS) reference material (Cola Drink Proficiency Testing Material 03119) was used as control during the analysis. Deionized water of conductivity $\leq 1.0\ \mu Scm^{-1}$ was used. HPLC grade acetonitrile and isopropanol were purchased from Scharlau, Spain.

HPLC methods. The HPLC methods (Shimadzu 20 series) include different combinations of analytical column and detector namely column amine and refractive index detector (HPLC-RID-AMINO), column hydrophilic interaction liquid chromatography (HILIC) and refractive index detector (HPLC-RID-HILIC), strong anion exchange (SAX) column and refractive index detector and (HPLC-RID-ANIONIC), column amine and diode array detector and (HPLC-DAD-AMINO) and column amine together with evaporating light scattering detector (HPLC-ELSD-AMINO). All combinations were tested in this study so as to determine the most suitable method for separation and quantification of sugars (fructose, glucose and sucrose).

Choosing the most suitable HPLC method. Parameters such as accuracy, reproducibility, sensitivity, volume of sample used, organic wastes generated and cost were applied in selecting the most suitable HPLC method for the quantification of the different natural sugars in soft drinks. Experimental design was used to obtain the best conditions for the analysis of the sugars in the soft drink. SPSS package 20.0 was used to determine factors which contribute the most to the response of the three sugars and to evaluate their interactions.

Calibration. Three different calibration curves were prepared for each of the sugars; fructose, glucose and sucrose. The different parameters namely gradient, intercept, coefficient of determination, mean response factor, limit of detection (LOD) and limit of quantification (LOQ), associated with the calibration curves were also reported and fructose was chosen as an example.

Method validation. Method validation was used to establish the performance characteristics of analytical methods [14].

System suitability test. The system suitability test was used to evaluate column chromatographic parameters and also column performance. A standard of known concentration was injected seven times under the same conditions and parameters investigated were peak areas, retention times, tailing factor and number of theoretical plates were evaluated for their % RSD (percentage relative standard deviation)

Precision check. Precision was assessed on three consecutive days using the prepared standard called QCCheck. The check was run seven times daily for three consecutive days. The mean areas obtained daily for each analyte was used to calculate the % RSD for fructose, glucose and sucrose.

Accuracy check. Accuracy was evaluated through percent recovery. A sample of soft drink was spiked with the QCCheck in the ratio of 1:1. By fortifying the soft drink, matrix effect also has been verified.

Robustness. Robustness was used to study small variations that occurred in the system during analysis due to change in conditions. The purpose was to identify factors (% acetonitrile in the mobile phase, Column oven temperature, flow rate, injection volume and autosampler temperature) that were most sensitive to change in conditions. Once the factors are identified, they can be controlled so as to prevent significance of deviation.

RESULTS AND DISCUSSIONS

Choosing the most appropriate HPLC method for the determination of total sugars in soft drink by experimental design. HPLC methods are considered as the best method used for the analysis of sugar in soft drinks for the various reasons such as they are sensitive, accurate, not time consuming, and a small amount of sample is needed (2 µL) [15]. However HPLC-RID-AMINO (Figure 1) was considered the best among all the HPLC methods employed in this work since it used lesser mobile phase (1.8 mL of Acetonitrile per sample) as compared to the HPLC-RID-HILIC and HPLC-RID-ANIONIC where 6 mL of acetonitrile per sample were used. Also better separation (resolution of greater than 2.5) of the sugars using HPLC-RID-AMINO was achieved with much better response (LOD for the three different sugars ranged from 0.22 to 0.38% w/v). Though HPLC-ELSD-AMINO (Figure 2) was most sensitive among all the methods, it was the most expensive, difficult to operate as it requires a source of constant nitrogen gas flow which rendered the method less stable. HPLC-DAD-AMINO is also very expensive and very little knowledge on its reliability was known, In addition, when FAPAS reference material was analysed using the five HPLC methods, HPLC-RID-AMINO gave the most accurate value. The theoretical value for FAPAS cola drink was 10.8, the % accuracy for each method was calculated.

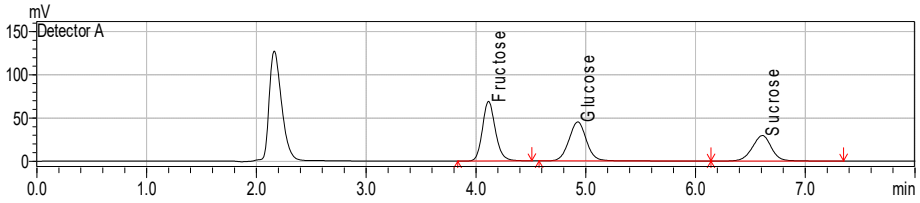

Figure 1. Separation of sugars using HPLC-RID-AMINO (ACN % 85, temperature 40 °C and flow rate 0.3 mL/min).

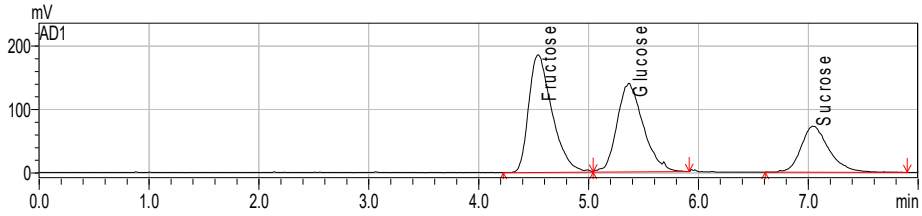

Figure 2. Separation of sugars using HPLC-ELSD-AMINO (ACN % 75, temperature 30 °C and flow rate 0.1 mL/min).

Interactions between set conditions. Now that HPLC-RID-AMINO revealed to be the most suited method for the separation of the three natural sugars, full factorial design (Table 3) was used to evaluate the best conditions in terms of column temperature (x_1), flow rate (x_2) and mobile phase ratio (x_3) that would give the optimum response for each sugar. FAPAS Reference Material was also used for this experiment. The three variables (x_1, x_2 and x_3) were varied accordingly (Table 1). All possible interactions were then considered through full factorial design. From the different interactions among the three parameters namely column oven temperature, flow rate and mobile phase ratio, the following coefficients have been computed for fructose, glucose and sucrose.

Table 1. The different conditions applied to HPLC.

Factors	Levels	Sign
Column oven temperature (^oC) x_1	Low = 30	-1
	Medium = 35	0
	High = 40	1
Flow rate (mL/min) x_2	Low = 0.1	-1
	Medium = 0.2	0
	High = 0.3	1
Mobile phase ratio (acetonitrile:water) x_3	Low = 75:25	-1
	Medium = 80:20	0
	High = 85:15	1

The regression equation for fructose was given by the equation:

$$y = 2600000 + 40000x_3 - 30000x_1 - 890000x_2 - 2970x_3x_1 + 28049x_2x_1 - 8950x_2x_3 + 15552x_1x_2x_3$$

Since x_2 showed the highest R % (24.52%), it indicated that flow rate gave the best response for fructose as compared to the other factors. The relationship was negative which meant that as flow rate decreases the response increases. The regression equation for the model glucose was given by the equation:

$$y = 2120000-300000x_3-220000x_1-570000x_2-86734x_1x_3+120101x_2x_1-178104x_2x_3+50948x_1x_2x_3$$

Likewise, flow rate determined the best response for glucose as compared to the other factors but in this case temperature and the mobile phase ratio also contribute to the response but to a lesser extent. The relationship was negative for all the three factors as in fructose. The regression equation for the model sucrose was given by the equation:

$$y = 2790000 + 40000x_3 - 11191x_1 - 960001x_2 - 1091x_1x_3 + 6943x_2x_1 - 30230x_2x_3 + 10509x_1x_2x_3$$

Again flow rate determined the best response for sucrose as compared to the other factors. The relationship is negative which means that as flow rate decreases the response increases. Briefly, from the statistics carried out it was observed that when flow rate, temperature and % of acetonitrile were set at their lowest levels, optimum conditions were reached for the separation of the three sugars (Figure 3). On the other hand, when the highest levels for the different conditions were set, poor sensitivity was observed.

Validation of HPLC-RID-AMINO. Having determined the optimum conditions, calibration curves for fructose, glucose and sucrose were constructed using reference standards. The coefficient of determination (R^2) was greater than 0.999 which showed good linearity. Validation of the HPLC-RID-AMINO was then undertaken by carrying out system suitability test, precision check, accuracy check and robustness.

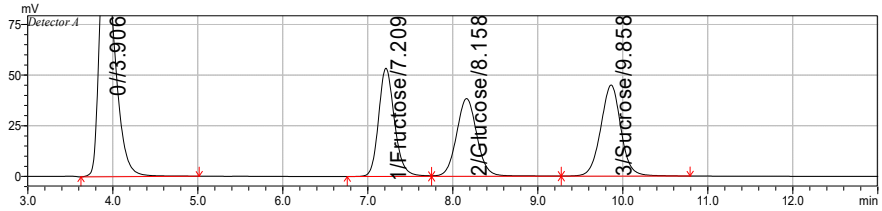

Figure 3. Chromatogram with conditions set at lowest levels (ACN % 75, temperature 30 °C and flow rate 0.1 mL/min).

System suitability test (SST). The HPLC column (amine column) for the HPLC-RID-AMINO was verified for its performance in terms of retention times, peak area, tailing factor and number of theoretical plates for each of the sugars using the FAPAS reference material containing 10.8 g % w/v of total sugars. The relative standard deviation (RSD) was used for the chromatography column test. The acceptance criteria (% RSD) were set as per AOAC performance requirements set for HPLC methods of analysis [14, 16]. Results obtained for one sugar (fructose) is as per Table 2. % RSD of less than one percent was obtained for all the parameters investigated for the three sugars and were within range as set by International conference on harmonisation ICH [16], implying that the column chromatography was suitable for the analysis of sugar in soft drinks.

Table 2. System suitability test for fructose.

Name	Retention times	Conc (%)	Peak area	Peak height	Tailing factor	Theoritical plate
RSD % criteria	< 2	< 2	< 2	< 2	< 2	> 2000
Fructose 1	3.83	3.16	2120492	129969	1.08	7335
Fructose 2	3.82	3.15	2115693	130210	1.26	7356
Fructose 3	3.83	3.15	2115585	131175	1.26	7361
Fructose 4	3.83	3.11	2090829	129440	1.27	7357
Fructose 5	3.83	3.09	2074817	130361	1.26	7390
Fructose 6	3.83	3.13	2106947	130177	1.26	7345
Fructose 7	3.83	3.14	2110209	130432	1.26	7340
Mean	3.83	3.13	2104939	130252	1.24	7355
Standard Dev.	0.0025	0.025	16376	523	0.069	
RSD %	0.065	0.789	0.778	0.402	-	-

Precision check. A standard check (QCChcek) containing 2% of fructose, 2% of glucose and 11% of sucrose was prepared and run 7 times daily over three consecutive days. The mean areas obtained daily over 3 days were used to compute the % RSD (Table 3). % RSD of less than 1% showed good precision as the acceptable criteria set was less than 5% [14].

Accuracy check. To study the matrix effect, the spiked solution (50% soft drink and 50% QCCheck) was injected. It was observed that the % recovery varied from 98.48 to 99.42% (Table 4). The criterion set for the % recovery was 98% to 102% [14].

Robustness. Robustness was used to determine whether the results of analysis were affected by small variations in conditions set for analysis. The factors chosen for the analysis were flow rate (A), % acetonitrile (B), volume injection (C), column oven temperature (D), and auto sampler

temperature (E) and the levels applied were as tabulated. Factor analysis was used to determine which parameter was the most susceptible to change while applying the different conditions, without considering the interactions of the five parameters. The following results were obtained while computing for the estimated % effect for each natural sugar (Table 5).

Table 3. Precision on three consecutive days.

	Sugars	Fructose	Glucose	Sucrose
QC Check	Criteria set RSD%	<5	<5	<5
	Day1	3689118	3360042	1855985
	Day2	3681917	3350338	1873068
	Day3	3708821	3391316	1890098
	Mean	3693285	3367232	1873050
	SD	13928	21414	17057
	RSD%	0.3771	0.6360	0.9106

Table 4.Accuracy Check using QC Check.

	50% QC check	50% soft drink	Theoretical yield	Actual yield	% Recovery
Fructose	1.0004	1.6712	2.6715	2.6668	99.82
Glucose	1.0010	1.8294	2.8303	2.8164	99.51
Sucrose	5.5236	1.8032	7.3268	7.2153	98.48
Total	7.5249	5.3038	12.8287	12.698	98.99

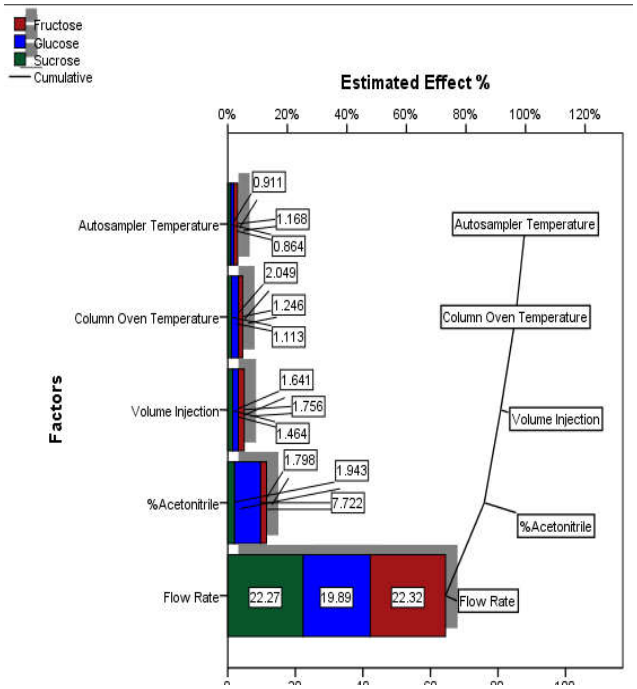

Figure 4. Pareto chart to indentify major causes.

Table 5. Estimated % effect of factors and Significance values.

	Fructose		Glucose		Sucrose	
Parameters	Estimated effect %	Sig. value	Estimated effect %	Sig. value	Estimated effect %	Sig. value
%ACN	1.798	0.004	7.722	0.000	1.943	0.029
Column oven temperature	1.246	0.039	2.049	0.034	1.113	0.197
Flow rate	22.32	0.000	19.89	0.000	22.27	0.000
Volume injection	1.756	0.005	1.641	0.085	1.464	0.093
Autosampler temperature	1.168	0.052	0.911	0.330	0.864	0.314

From the data generated through factor analysis, a pareto chart was plotted (Figure 4) so as to identify the vital few that is the "20%" causes which if controlled can solve 80% of the problems.

From the five factors analysed for robustness, flow rate was the most affected by a change in condition since the percentages of estimated effect for fructose, glucose and sucrose were much higher compared to those for auto sampler temperature, column oven temperature, volume injection and % acetonitrile. Hence variation in the flow rate needs to be controlled throughout analysis by HPLC.

CONCLUSIONS

Five HPLC methods were developed for the analysis of sugars, namely; fructose, glucose and sucrose in soft drinks and lemonades. The best separation was obtained with HPLC-RID-AMINO method. Three main factors, namely flow rate, concentration of acetonitrile in the mobile phase and column oven temperature for the determination of each of the sugars by the HPLC method were analysed individually using experimental design. The optimum condition for the three factors was obtained and retained for this method. From the experiment it was observed that when the flow rate was increased from 0.1 mL/min to 0.3 mL/min, the concentration of the three sugars was reduced by almost 20%. Glucose in addition to flow rate was also subsequently affected by the other two factors, the concentration of acetonitrile in the mobile phase and the oven temperature. An increase in concentration of acetonitrile in the mobile phase from 75% to 85% causes a reduction in the concentration of glucose by 7%. An increase in the temperature of oven from 30 ^{o}C to 40 ^{o}C caused a further reduction in the concentration of glucose by 2%. These findings could be used to improve the final results of the three sugars by increasing the sensitivity and improving the robustness of the method.

The method was validated according to International Conference on Harmonisation (ICH) related to system suitability test taking into consideration parameters as precision, accuracy check and robustness. The method was then tested during a proficiency test, inter-laboratory testing using FAPAS certified reference material and was found to give accurate results. The HPLC-RID-AMINO method was then used to analyse fructose, glucose and sucrose in samples of soft drinks and lemonades to determine the total concentration of each sugar present in these beverages as percent weight by volume (% w/v) for authority to apply the sugar tax based on these results. From the statistical analysis carried out including method development and validation, we conclude that each stage considered is important to achieve accurate results for all the three sugars considered so far.

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