THE USE OF TITRIMETRIC, NELSON SOMOGYI AND HPLC METHODS FOR THE ANALYSIS OF CASHEW APPLE JUICE FERMENTATION BROTHS

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ABSTRACT

In cashew apple juice fermentation to produce wine and vinegar, analysis of organic acids and sugars in fermentation broths is very important, due to the fact that optimum conditions of fermentation could only be established from results obtained on monitoring the concentrations of those components during the fermentation process. Analysis of organic acids by titrimetric method and analysis of sugars by Nelson-Somogyi method only give a total amount of acids and sugars. HPLC is one of the promising method for determining the acids/sugars individually, although this method needs costly facilities such as columns and solvents. In this work, organic acids were separated by HPLC on a μ-Bondapak C_{18} column using aqueous solution of 2% $NH_4H_2PO_4$ as the mobile phase, while sugars were separated on silica-based column using an eluent containing a polyamine reagent. In this investigation the results of determination of organic acids by titrimetric method and those of sugars by Nelson-Somogyi method were compared respectively with the results of individual organic acids and sugars obtained from the HPLC methods. It was found that for organic acids, results of the determination using the titrimetric method is correlated linearly with the results of acetic acid obtained by the HPLC methods. The same results were obtained for total and reducing sugars determination by the Nelson-Somogyi and individual sugar by the HPLC methods. The regression equation obtained for each of the organic acids and sugars can be used for the estimation of each of the respective components present in the cashew apple juice fermentation broths based on the results obtained from both titrimetric and Nelson-Somogyi methods. For routine monitoring of large number of fermentation broth samples, the proposed method was found to be a better alternative to the more costly HPLC method.

INTISARI

Dalam proses fermentasi sari buah jambu mete untuk menghasilkan anggur dan cuka, penentuan asam-asam organik dan gula sangat penting. Kondisi optimal proses fermentasi tersebut hanya dapat ditentukan berdasarkan data hasil analisa yang diperoleh pada saat memantau kandungan komponen-komponen tersebut diatas, selama proses berlangsung. Analisa asam-asam organik dengan metoda titrimetri dan analisa gula dengan metoda Nelson-Somogyi, hanya dapat memberikan data mengenai jumlah total asam organik dan gula. Metoda HPLC dapat digunakan untuk menentukan berbagai jenis asam organik dan gula secara individual, meskipun metoda ini membutuhkan sarana yang mahal seperti kolom dan pelarut. Dalam percobaan ini, asam-asam organik dipisahkan melalui metoda HPLC pada kolom μ-Bondapak C_{18} menggunakan larutan 2% $NH_4H_2PO_4$ sebagai fasa gerak. Sedangkan berbagai jenis gula dipisahkan pada kolom dari bahan dasar silika menggunakan fasa gerak yang mengandung pereaksi poliamina. Pada percobaan ini hasil analisa asam-asam organik dengan metoda titrimetri dan hasil analisa gula dengan metoda Nelson-Somogyi dibandingkan terhadap hasil analisa asam organik dan gula secara individual dengan metoda HPLC. Ditemukan bahwa untuk asam-asam organik, hasil yang diperoleh dari metoda titrimetri mempunyai korelasi yang linier terhadap hasil analisa asam asetat menggunakan HPLC. Hal yang sama ditemukan pada penentuan gula total/gula pereduksi dengan metoda Nelson-Somogyi dan penentuan gula secara individual dengan metoda HPLC. Persamaan garis regresi yang diperoleh untuk setiap asam organik dan gula dapat digunakan untuk memperkirakan kandungan setiap komponen yang terdapat dalam campuran hasil fermentasi sari buah jambu mete. Untuk penentuan rutin sejumlah besar contoh campuran hasil fermentasi, metoda yang diusulkan ini dapat merupakan metoda alternatif dari metoda HPLC yang jauh lebih mahal.

INTRODUCTION

In cashew apple juice fermentation to produce wine and vinegar, analysis of organic acids and sugars in fermentation broths is very important, since the optimum condition of fermentation could only be established from results obtained on monitoring the concentrations of those components during the fermentation process.

The development of methods for the analysis of organic acids and sugars in fermentation broths has received considerable attention. Many analytical procedures have been reported including those of conventional as well as instrumental methods.

Analysis of organic acids and sugars in fermentation broths is a difficult task. Conventional methods used in this type of analysis is time consuming and gives only the total amount of organic acids and sugars. It seems impossible to determine the individual acids and sugars by conventional methods.

Ion-exchange chromatography at elevated temperature (70°C) has been used in the separation of organic acids in mixtures, but this method is time consuming and the ion-exchange columns employed may easily become poisoned (1,2). Among HPLC methods, the use of a μBondapak C_{18} column with an aqueous 2% $NH_4H_2PO_4$ solution adjusted to pH 2.8 (using H_3PO_4) as a mobile phase has also been reported (3). The latter method was then adopted in our laboratory to allow the simultaneous determination of several organics acids, saccharose and ethanol in cashew apple fermentation broths (4,5). However, it was found that in this chromatographic system, glucose and fructose have the same retention time, too close to the solvent peak and very near to that of tartaric acid. Therefore, the above method allows separation of the monosaccharides (fructose, glucose) from disaccharides (saccharose) but not from each other.

Sugars separation by HPLC has also been achieved on silica column that had been chemically modified into a basic form. Chemically bonded amine columns such as Lichrosorb-NH_2, μ-Bondapak Carbohydrate and Partisil PAC have been widely used in HPLC for sugars separation using acetonitrile-water mixtures as mobile phases (6,7,8,9). However, these columns tend to deteriorate with prolonged use, with the loss of their amine function, possibly due to gradual formation of Schiff bases. This *on-column* reaction, has resulted in losses of reducing sugars being analyzed. This problem has been avoided when another column (e.g. Diol, C_{18}) or a non reactive amine column such as Nucleosil [$N(CH_3)_2$] are used (6). Examination of the chromatographic properties of sugars on Diol and C_{18} Columns has been carried out (10), but there have been still many technical problems associated with the method. Later WATERS produced Silica Amine Modifier (SAM) reagent, containing a non-reactive polyfunctional amine (11), which were able to modify silica column by adding the modifier to the eluent and use the mixture to impregnate the silica column *in-situ*. Adoption of this method

in our laboratory resulted in a good separation of eight sugars (12).

The aim of the present work is to develop methods suitable for rapid and economical determination of individual organic acids and sugars in cashew apple juice fermentation broth. A study was carried out on the application of titrimetric method for organic acids, Nelson-Somogyi method for sugars, and HPLC method for both organic acids and sugars.

EXPERIMENTAL

Reagents

Water used for the preparation of mobile phases was purified using a Millipore (Bedford, M.A., USA) Milli Q Water purification system. SAM reagent-1 was from WATERS. Mobile phases and standard solutions were filtered through a 0.45 µm membrane filter, while mobile phase were degassed in an ultrasonic bath before use. All standards and chemicals namely tartaric, malic, ascorbic, lactic, acetic, citric, fumaric acids, glycerol, rhamnose, xylose, fructose, glucose, sucrose, maltose, lactose, phenolphthalein and sodium hydroxide were of reagent grade from E.Merck.

For the separation of organic acids, an aqueous 2% $NH_4H_2PO_4$ solution adjusted to pH 2.8 with H_3PO_4 was used as a mobile phase. **For the separation of sugars,** a silica cartridge was initially conditioned before use by pumping 500 ml acetonitrile-water mixture (385:15) to which 5 vials of Waters SAM reagent-1 had been added, through the column. The mobile phase was prepared by adding one vial of SAM reagent 1 to a mixture of acetonitrile-water (770:210).

Instrumentation

A Beckman DU-50 UV-VIS spectrophotometer and a liquid chromatograph (Waters Associates) consisting of a model 6000A solvent delivery system, Model U6K Universal Injector and Model R-401 Differential Refractometer were used. For the separation of organic acids a µBondapak C_{18} column (30 cm x 4 mm ID, Waters Associates) was used and the flow rate of the mobile phase was adjusted to 1.5 ml/min. For the separation of sugars, radial-Pak Silica cartridge (10 cm x 8 mm I.D.) in WATERS RCM-100 radial compression module was used and the flow rate of the mobile phase was adjusted to 3 ml/min. Peak areas were measured by using a Waters Data Module and a Spectra Physics (SP 4920) Integrator.

Method

Total acids was determined by titrating an aliquot of fermentation broth with 0.01 N standard NaOH solution using phenolphthalein as visual indicator.

Reducing sugars and total sugars were determined by Nelson-Somogyi spectrophotometric method (13). A calibration curve was made by preparing glucose standard solutions with concentrations ranging from 0.01 to 0.50 mg/ml and treating them as sample solutions. The wavelength used for absorbance reading was 520 nm.

For organic acids and sugars analysis using the HPLC method, aqueous standard solutions for each compound were prepared individually and chromatographed separately in order to determine the individual retention times. Mixed standard solutions containing several organic acids and sugars were prepared and chromatographed after the individual retention time had been established. Calibration curves were prepared by chromatographing mixed standard solutions. The injection volume was 20 µl for each standard solution. All solutions were filtered before injection into the column.

RESULTS AND DISCUSSION

Calibration Curve for Spectrophotometric (Nelson-Somogyi) Method.

The calibration curve of Nelson-Somogyi method shows a straight line (Figure 1) with correlation coefficient of 0.9972, indicating its reliability for quantitative determination.

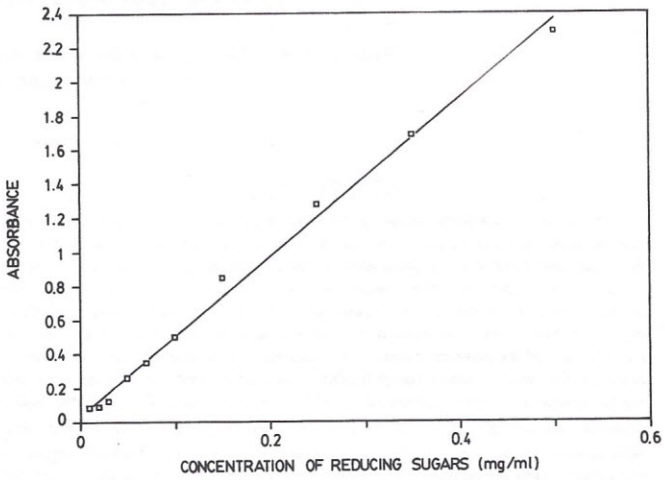

Figure 1: Calibration Curve for Reducing Sugars

Elution Profiles and Calibration Curves for the HPLC Method.

The elution profiles of organic acids and sugars separation are shown in Figure 2 and 3 respectively. The linearity of the

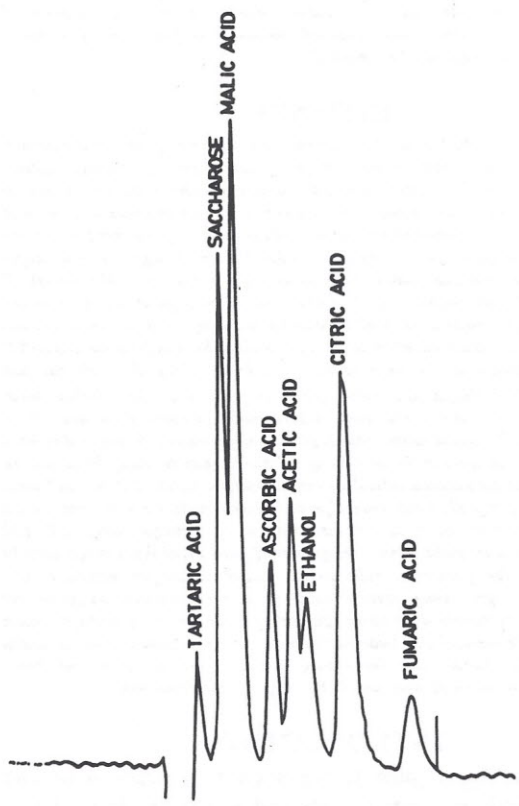

Figure 2: Elution Profile of Organic Acids Separation

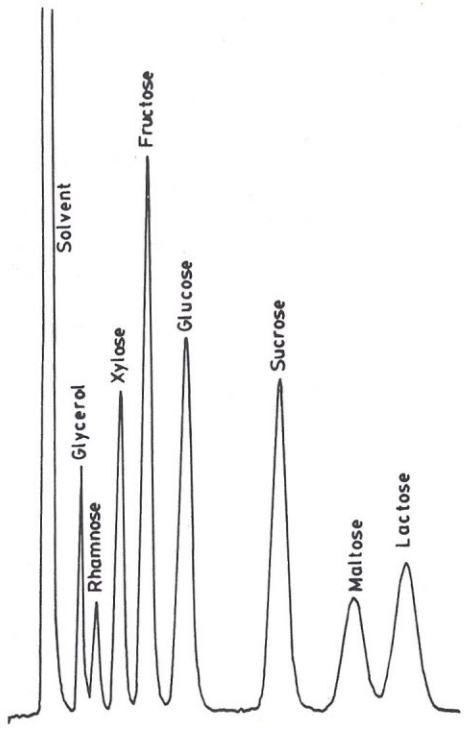

Figure 3: Elution Profile of Sugars Separation

calibration curve was tested by chromatographing a series of mixed organic acids or sugars standard solutions. Linear calibration plots for several organic acids are observed over the range 2-100 µgr of injected solutes (Figure 4). Results obtained from HPLC analysis shows that glycerol, fructose, glucose and sucrose are present in the fermentation broth (Figure 5).

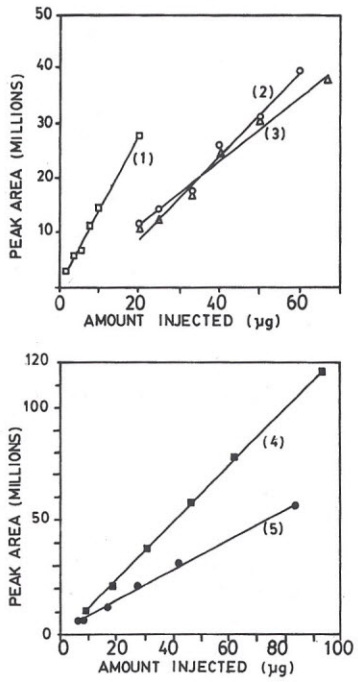

Figure 4: Calibration Curves for Organic Acids
Tartaric Acid (1) r = 0.9966;
Malic Acid (2) r = 0.9934;
Citric Acid (3) r = 0.9874;
Fumaric Acid (4) r = 0.9998 and
Acetic Acid (5) r = 0.9981.

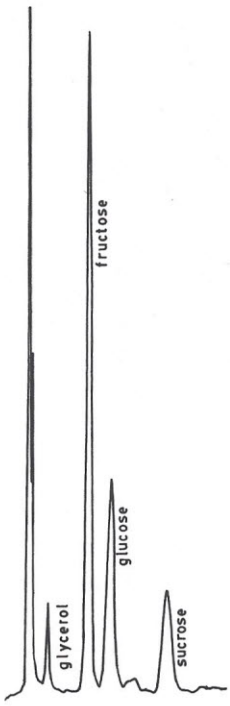

Figure 5: Chromatogramme obtained from the HPLC of Cashew Apple Fermentation Broth.

Linear calibration plots for glycerol, glucose, fructose and sucrose are observed over the range of 150-1300 µg of injected solutes (Figure 6).

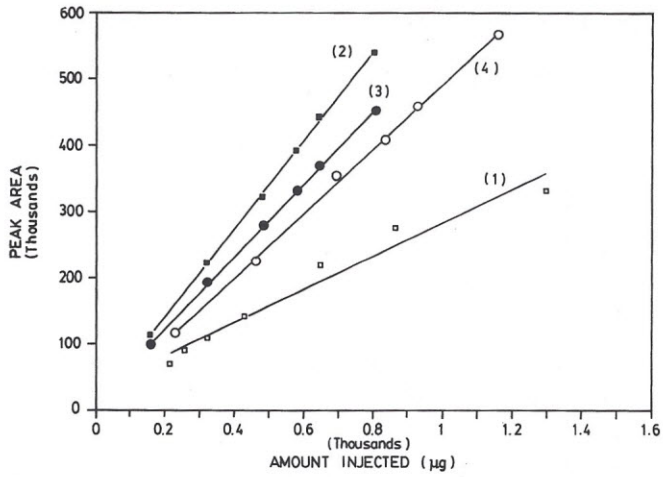

Figure 6: Calibration Curves for Glycerol and several Sugars
Glycerol (1) r = 0.9608; Fructose (2) r = 0.9992
Glucose (3) r = 0.9992; Sucrose (4) r = 0.9986.

Regression analysis of peak areas of organic acids and sugars demonstrated a linear relationship with near zero intercept and with correlation coefficient between 0.9608 - 0.9998.

Analysis of fermentation broths samples.

The determination of organic acids by titrimetric and HPLC methods was carried out on three groups of samples, each of them consisted of 10 samples. For the determination of sugars, 22

samples were analysed both by Nelson-Somogyi and HPLC methods. The analysis results obtained are shown in Table 1 and 2.

Table 1. Total Acid Concentrations obtained by Titrimetric Method and Acetic Acid Concentrations obtained by HPLC.

	Concentration of Total Acids obtained by Titrimetric Method			Concentration of Acetic Acid obtained by HPLC Method		
Sample Number	Serie A % (W/V)	Serie B % (W/V)	Serie C % (W/V)	Serie A % (W/V)	Serie B % (W/V)	Serie C % (W/V)
1	0.47	0.69	0.66	0.00	0.00	0.00
2	0.76	1.92	1.26	0.66	1.53	1.04
3	1.18	2.71	2.02	1.06	2.22	1.31
4	1.65	3.43	2.73	1.53	3.12	2.45
5	2.06	4.13	3.32	1.87	4.07	3.65
6	2.48	4.33	3.44	2.50	4.56	3.70
7	3.23	4.37	3.83	3.24	4.17	4.03
8	3.34	4.45	3.84	3.25	4.39	3.71
9	3.70	4.54	3.86	3.55	4.43	3.95
10	3.81	4.76	3.97	3.59	4.70	3.59

Table 2. Reducing Sugars and Total Sugars Concentrations obtained by Nelson-Somogyi Method and Individual Sugars Concentrations obtained by HPLC.

	Nelson-Somogyi Method		HPLC			
Sample Number	Reducing Sugars % (W/V)	Total Sugars % (W/V)	Glycerol % (W/V)	Glucose % (W/V)	Fructose % (W/V)	Sucrose % (W/V)
1	14.16	15.94	–	5.95	5.86	1.85
2	15.82	15.62	–	6.87	6.73	–
3	16.40	15.35	–	6.30	6.70	–
4	15.60	15.04	–	6.11	6.49	–
5	13.86	13.71	–	5.74	6.56	–
6	10.30	10.74	0.32	4.19	5.68	–
7	12.28	11.73	0.39	4.20	5.94	–
8	9.23	9.88	0.42	4.15	6.05	–
9	11.47	10.22	0.40	3.58	5.80	–
10	9.80	11.05	0.58	3.56	5.81	–
11	9.89	8.65	0.55	2.90	5.40	–
12	9.10	7.59	0.58	2.55	5.27	–
13	8.77	7.84	0.55	2.45	4.96	–
14	8.09	7.00	0.53	1.98	4.58	–
15	6.79	6.10	0.54	1.68	4.66	–
16	7.16	6.50	0.57	1.93	4.73	–
17	6.51	5.81	0.46	1.51	4.46	–
18	6.19	5.34	0.64	0.91	4.38	–
19	5.23	4.94	0.67	0.95	4.20	–
20	5.56	5.03	2.90	0.90	3.97	–
21	5.39	4.83	6.58	0.70	3.89	–
22	4.92	4.34	7.83	0.68	3.78	–

Comparison of Results.

Results of HPLC analysis of fermentation broth samples showed that only acetic acid was present in sufficient amount to be detected. Total acid concentrations of several fermentation broth samples obtained by titrimetric method are thus compared with the concentrations of acetic acid obtained by HPLC. Similarly, reducing sugar and total sugar concentrations obtained by Nelson-Somogyi method are compared with individual sugar concentration obtained by HPLC. The calculated regression equations are listed in Table 3 and 4. The concentration of total

Table 3. Correlation of Results between Titrimetric Method and HPLC in Acetic Acid Determination.

Method	Solute Tested	Experiment	R^2	Regression Equation
Titration vs HPLC	Acetic Acid			Y = Titration X= HPLC
		A	0.9904	Y = 0.9484 X + 0.2545
		B	0.9932	Y = 0.8507 X + 0.7079
		C	0.9608	Y = 0.8037 X + 0.6878

Table 4. Correlation of Results between HPLC and Nelson-Somogyi Method in Sugars Determination

Method	Solute Tested	R^2	Regression Equation	Respective Figure
HPLC vs Nelson-Somogyi Method			Y = HPLC X = Nelson-Somogyi	
	Total Sugars	0.9833	Y = 0.7782 X + 1.3344	8 A
	Reducing Sugars	0.9515	Y = 0.7837 X + 0.8698	8 B
	Reducing Sugars vs Fructose	0.8955	Y = 0.2454 X + 2.8975	8 C
	Reducing Sugars vs Glucose	0.8546	Y = 0.4715 X - 1.2613	8 D

acids found by titrimetric method are well correlated (R^2 between 0.9608 and 0.9932) to the concentration of the acetic acid found by HPLC (Figure 7). In every case, acetic acid values obtained from HPLC method are lower than the corresponding total acid values obtained from titrimetric method. The average slope obtained from analysis of the three batches of fermentation broth samples is 0.8676 with a standard deviation of 0.0778 and coefficient of variance of 8.5%. Intercepts from each of the three regression equations (i.e. from experiment A, B and C) have different values, because in the titrimetric method the sodium hydroxide standard solution reacts with other organic acids in addition to acetic acid, and possibly the concentrations of the other acids present in different fermentation broth samples tested are different.

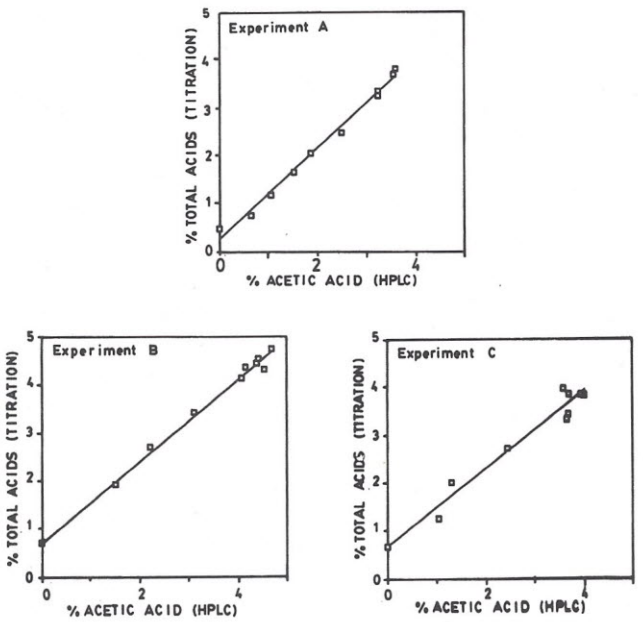

Figure 7: Correlation of results between Titrimetric method and HPLC in Acetic Acid Determination, obtained from three experiments, using three groups of different samples.

The HPLC analysis of a fermentation broth sample shows that only glycerol, fructose, glucose and sucrose are present in the mixture (Figure 5). Glycerol could not be determined by the Nelson-Somogyi method, because it has no reducing group. Due to this reason, the concentration of glycerol in the fermentation broth is not considered in the calculation.

In the determination of sugars, the concentration of total sugars obtained by Nelson-Somogyi method are compared with the sum of the concentration of glucose, fructose and sucrose determined by HPLC method (Figure 8A). In the other case, the concentration of reducing sugars obtained by Nelson-Somogyi method are compared with the sum of only the concentration of glucose and fructose determined by HPLC method (Figure 8B). The results have a good response and the concentration of total sugars and reducing sugars nearly the same, because there were no more sucrose in the fermentation broth samples analysed.

If the individual concentration of fructose and glucose obtained by HPLC is compared each with the concentration of the total reducing sugar obtained by the Nelson-Somogyi method, good correlations are found (Figure 8C and 8D). Therefore the regression equations obtained may be used for calculation of concentration of the individual sugars (fructose and glucose) from the concentration of the total reducing sugars obtained by Nelson-Somogyi method.

CONCLUSION

Based on the observations and results obtained from this investigation, it is concluded that in the analysis of organic acids and sugars of cashew apple fermentation broth, HPLC method has more advantages compared with the titrimetric method and Nelson-Somogyi method, since by using the HPLC method each acids/sugars can be determined separately from one another.

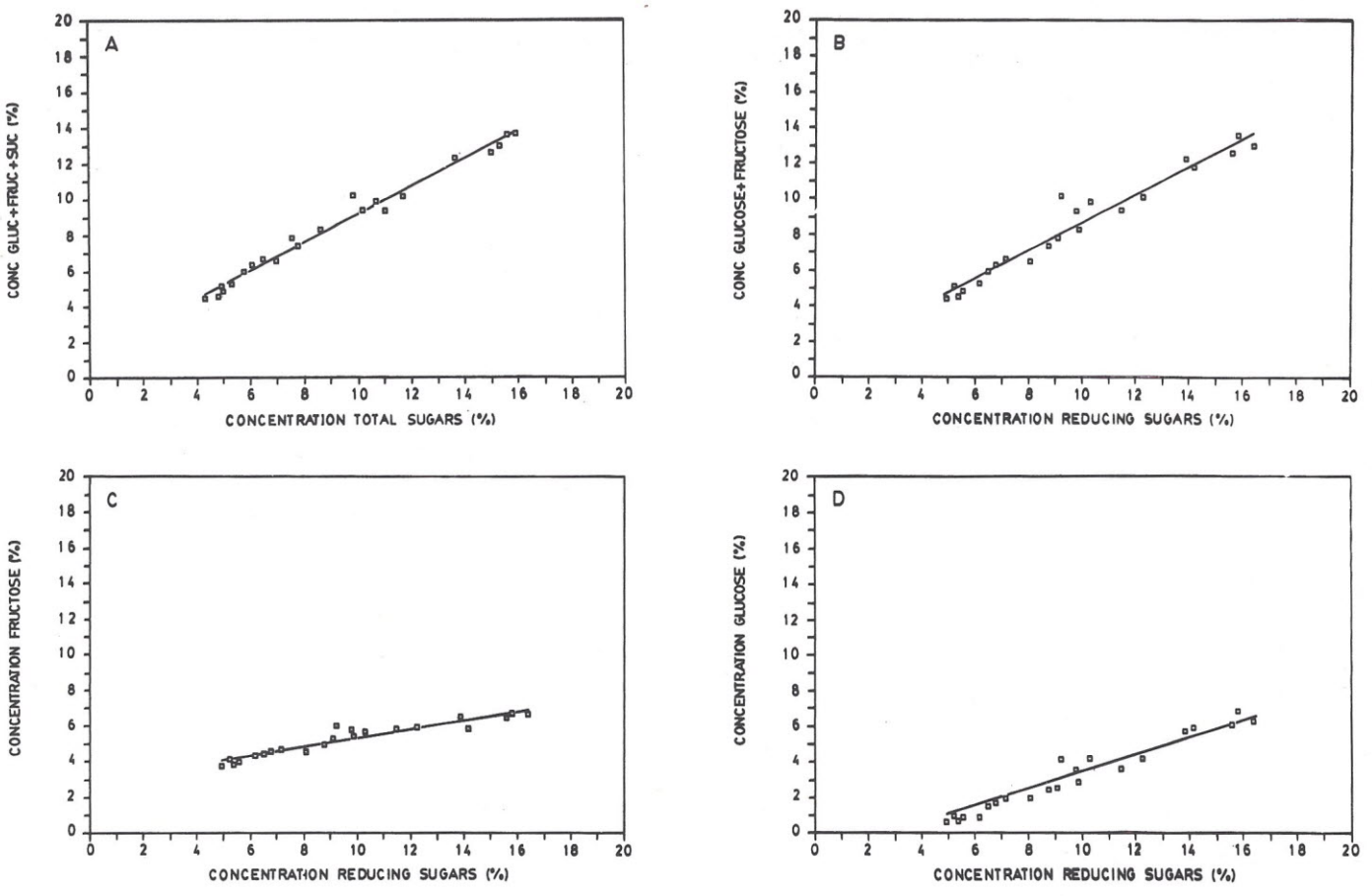

Figure 8: Correlation of results between HPLC and Nelson-Somogyi Method in Sugars Determination

However, HPLC method needs costly facilities such as columns and solvents and therefore HPLC analysis of all fermentation broth samples is highly undesirable. To avoid the high running cost, the regression equation obtained from the comparison of results obtained by HPLC method and those obtained by titrimetric/Nelson-Somogyi method may be used as an inexpensive alternative for routine estimation of individual sugar and organic acid in cashew apple fermentation broth samples. It is proposed that the HPLC method be used only for the analysis of selective samples only.

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