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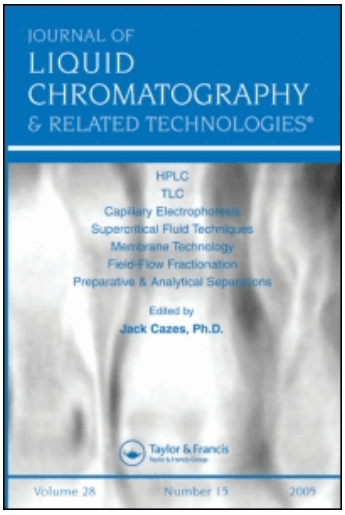

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Evaluation of Calibration Table and Calibration Averaging Routines for Quantitative Determination of Sucrose, Glucose, and Fructose Using High Performance Liquid Chromatography

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EVALUATION OF CALIBRATION TABLE AND CALIBRATION AVERAGING ROUTINES FOR QUANTITATIVE DETERMINATION OF SUCROSE, GLUCOSE, AND FRUCTOSE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Evaluation of Calibration Table and Calibration Averaging Routines developed by Waters Associates for quantitative determination of sucrose, glucose and fructose using Waters Associates Sugar Analyzer is discussed. Both routines are sufficiently accurate and offer some advantages over previously-described Lane-Eynon Method of determination of reducing sugars by means of Fehling Solution with Methylene Blue as internal indicator. However, great care must be taken to prevent sucrose inversion on Sugar-PAK I column, which could otherwise render the quantitation of sugars useless.

INTRODUCTION

The Lane-Eynon (LE) Method[1] is one of the ICUMSA[2] recommended methods to determine concentration of reducing sugars after

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inversion. Because of it's laboriousness and other shortcommings[3] attempts were made in the past to analyze samples of sugars and molasses by high performance liquid chromatography (HPLC) and compare the results with the LE Method. Test determination of sugars by both methods, conducted during a two months period in our laboratory, indicated that the results obtained by HPLC did not compare well with the LE results when using the Calibration Table Routine, as described in Waters Associates Manual[4]. In order to find out if any improvement of HPLC results can be attained, Calibration Averaging Routine (supplied by Waters Associates as a supplement to Data Module M 730 Operator's Manual) was tried and compared to the results obtained by the Calibration Table Routine.

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MATERIALS AND METHODS

Chromatographic Conditions

Mobile Phase:	20 mg of calcium propionate/1L deionized water (fresh daily)
Pump, Flowrate:	0.5 mL of mobile phase/min.
Mobile Phase, Sparging:	5 min./pure helium, prior to use
Pump, Pressure:	600 psi to 900 psi
RI Detector, Attenuation:	8x
Data Module:	P_1 (large pen) at 20, P_2 (small pen) at 0
Chart Paper Speed:	0.5 cm/min.
Sample, Standard, Dilution:	2.5 g to 7.5 g/500 mL (5 to 15 g/L), in mobile phase

Corn Syrup 42 DE, Standard,

Dilution: 1 g/50mL (20 g/L), in mobile phase

Sample, Standard, Filtration: 1x, Waters Associates Clarification Kit

Injection Size: 5 µL

Run Time: 13 min.

Column Temperature: 90°C

Air Pressure, Autosampler: 40 psi

Sample and Standard Solutions

	Weight (g)			Relative Concentration*		
	F	G	S	F	G	S
Sample 1/500 mL mobile phase	0.5	0.5	1.5	10	10	30
Sample 2/500 mL mobile phase **	1.0	1.0	3.0	20	20	60
Sample 3/500 mL mobile phase	1.5	1.5	4.5	30	30	90
Standard/500 mL mobile phase **	1.0	1.0	3.0	20	20	60

* relative to Standard solution (combined concentration of S, G, F in standard is considered 100); the figures reflect absolute amounts of the individual sugars in sample.

** Sample #2 and "Standard" are identical solutions.

Equipment

Waters Associates Sugar Analyzer I Liquid Chromatograph, consisting of:

a) Model M-45 Solvent Delivery System,

b) Model WISP 710 B Automatic Sample Injection System,

c) Model R 401 Differential Refractometer,

d) Model Sugar-PAK I Column, stainless steel, 30 cm long, 1 cm O.D.,

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e) Model M 730 Data Module,

f) Model III Temperature Control Unit.

Chemicals, Supplies

a) D-Fructose, Fisher Scientific, Reagent Grade, (F),

b) D-Glucose, Fisher Scientific, Certified, A.C.S., (G),

c) Sucrose, Fisher Scientific, Certified, A.C.S., (S),

d) 42 DE Corn Syrup, Waters Associates,

e) Water, de-ionized, bacteria free (using Model MILLI-R/Q System Water Purifier by Millipore), contaning 20 mg/L of calcium propionate,

f) Waters Associates Sample Clarification Kit, Aqueous, filter pore size 0.45 μm,

g) Helium gas, pure,

h) Air, "Zero Gas".

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Chromatographic Procedure

Sugar-PAK I column was conditioned according to Waters Associates Manual [5], until the corn syrup chromatogram was acceptable and the sucrose peak was sharp. Sugar Analyzer was equilibrated for approximately 30 minutes by pumping the mobile phase. The standard was repeatedly injected, until reproducible amounts for S, G, F were observed (the last three injections varied less than 5 Area %). Calibration Table was created according to Data Module Manual and the samples 1, 2, 3 (3 repeats each were chromatographed twice, using WISP 710 Autosampler (vials #1, 2, 3 in carousel's positions 1, 2, 3) in Auto Mode. All found results are presented in Table 1.

TABLE 1
CALIBRATION TABLE

Sample #	Amount Found: Relative*** Concentration (and Area %, in brackets)		
	S	G	F
*	30.16(59.47)	10.31(20.33)	10.24(20.18)
1	30.18(59.37)	10.22(20.11)	10.42(20.50)
	29.43(58.76)	10.25(20.45)	10.41(20.78)
	58.86(59.96)	19.52(19.88)	19.78(20.15)
2	59.83(59.80)	19.94(19.93)	20.27(20.25)
	59.45(59.69)	20.02(20.09)	20.13(20.21)
	90.54(59.64)	30.67(20.20)	30.59(20.15)
3	90.28(59.86)	30.06(19.93)	30.47(20.20)
	90.44(60.13)	29.73(19.76)	30.22(20.09)
**	30.38(59.36)	10.54(20.59)	10.26(20.04)
1	30.16(59.15)	10.56(20.71)	10.26(20.12)
	30.39(59.89)	10.10(19.90)	10.25(20.19)
	60.54(59.96)	19.86(19.67)	20.56(20.36)
2	60.20(59.18)	20.14(20.06)	21.10(20.74)
	59.46(59.46)	20.68(20.67)	19.85(19.85)
	91.33(59.58)	30.98(20.21)	30.96(20.19)
3	91.43(59.92)	30.48(19.97)	30.67(20.09)
	91.59(59.68)	30.92(20.14)	30.95(20.16)

Run #	Average Area % ± SD		
	S	G	F
First	59.63±0.40	20.08±0.22	20.28±0.22
Second	59.58±0.31	20.21±0.37	20.19±0.25
First & second runs combined	59.60±0.35	20.14±0.30	20.24±0.23

* first run
** second run (duplicate)
(both runs are based on the same Calibration Table and the same solutions are used throughout).
*** see note in para. Sample and Standard Solutions (*).

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Calibration Averaging Routine was followed according to Data Module Manual. Carousel of the autosampler was loaded with 8 vials containing standard and samples in the following sequence: Standard, Samples 1,2,3, Standard, Samples 3,2,1,. The autosampler (in AUTO mode) was set for all 8 vials the same: number of injections (3), volume (5 uL), time (13 min.). Total time for the sequence was 5 hours 12 min. The Data Module was programmed in the following manner (Calibration Table created previously was used): # of injections of each sample = 3, # of injections of each standard = 3, # of samples to be analyzed before recalibration takes place = 3 and # of cycles = 0. The autosampler then proceeded to inject the standard (3x) from the vial #1. Response factors generated were averaged and used in previously created Calibration Table (first calibration). Samples 1,2,3 from vials #2,3,4 were injected (each 3x) and their compositions were reported (First Run). The autosampler then injected the standard from vial #5 (3x). Response factors generated were averaged and used for the second calibration. Samples 3,2,1 from vials #6,7,8 were injected (each 3x) and their compositions were reported (Second Run). All results are presented in Table 2.

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RESULTS AND DISCUSSION

Three repeats of samples 1,2,3, were chromatographed twice (two runs) using both Calibration Table and Calibration Averaging

TABLE 2
CALIBRATION AVERAGING ROUTINE

Sample #	Amount Found: Relative*** Concentration (and Area %, in brackets)		
	S	G	F
* 1	30.60(59.82)	10.30(20.14)	10.24(20.02)
	30.94(60.02)	10.53(20.42)	10.08(19.55)
	30.43(60.03)	10.22(20.16)	10.03(19.79)
2	60.26(60.33)	19.69(19.71)	19.94(19.95)
	60.12(59.54)	20.48(20.28)	20.37(20.16)
	60.40(59.58)	20.66(20.37)	20.32(20.04)
3	91.29(60.23)	30.45(20.09)	29.82(19.67)
	91.09(59.92)	31.11(20.46)	29.81(19.16)
	91.44(59.85)	31.15(20.39)	30.17(19.74)
** 1	89.59(60.19)	29.59(19.88)	29.65(19.92)
	89.82(60.22)	29.64(19.87)	29.69(19.90)
	90.06(60.00)	29.81(19.86)	30.22(20.13)
2	60.36(60.24)	19.78(19.74)	20.05(20.01)
	60.44(60.11)	20.01(19.89)	20.10(19.98)
	59.82(59.82)	20.25(20.24)	19.94(19.93)
3	29.79(59.67)	9.98(20.00)	10.14(20.31)
	28.91(59.18)	10.04(20.56)	9.89(20.24)
	29.80(59.57)	10.18(20.34)	10.05(20.08)

Run #	Average Area % ± SD		
	S	G	F
First	59.92±0.26	20.22±0.23	19.79±0.31
Second	59.89±0.36	20.04±0.27	20.06±0.15
First & second runs combined	59.91±0.31	20.13±0.26	19.92±0.27

* first run (run after first calibration)
** second run (run after second calibration)
(both runs are based on the same Calibration Table and the same solutions are used throughout).
*** see note in para. Sample and Standard Solutions (*).

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Routine methods. The results are presented in Tables 1 and 2. Relative concentrations of S, G, and F varied slightly from expected values, which are mentioned earlier. Area %'s (tabulated values in round brackets) of S, G and F, totaling of necessity for each sample to 100% were averaged and standard deviations (SD) were calculated. Calculated average Area % values for each run and combined runs are also presented in Tables 1 and 2. The comparison of average Area % values for both Calibration Table (T) and Calibration Averaging Routine (AR) has shown that they differed from expected values as much as 0.42%(T) and 0.22%(AR) for individual run and 0.40%(T) and 0.13%(AR) for both runs combined. Standard Deviation values were as high as 0.40(T) and 0.36(AR) for individual run and 0.35(T) and 0.31(AR) for both runs combined. There is very little difference in the Standard Deviation for both routines (0.29 and 0.285 on an average, overall, respectively). This is quite understandable considering that the SD reflect primarily the precision, that is determined by the reproducibility of the injection volume and of the area integration and should be independent from the calibration procedure. However, consistent improvement in the accuracy of the determination, expressed as the closeness of the averaged found results to the expected values, is observed. Because both routines exceed significantly the instrument specifications for reproducibility (± 5%), the improvement, in absolute term (0.01 to 0.31% on the grand means for individual components) is seemingly minor, but positive in all cases, favouring the AR routine. The accuracy of both routines is well below 1% (mean, 0.3%).

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While the mean differences between expected and found values of Area % for all three sugars in one sample must, by the way of calculation, always be zero, the absolute values of those differences were calculated and averaged (Table 3). The confidence ranges (95%) of the means or averages are 0.11 to 0.42% and 0.06 to 0.27% for the Calibration Table and Calibration Averaging Routines, respectively. The average of the ranges, as well as the found value of Student's statistics T=2.05, indicates that there is no evidence to demonstrate significant difference in the two routines.

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The Area % calculation method that eliminates errors due to extraneous (non-calibrated) peaks is very convenient for the evaluation of routine's performance because it allows for easy grand averaging. It is applicable for solutions of pure compounds only. For routine sample analysis, in particular the syrups and molasses analysis, the "relative concentrations" (i.e., the actual percentage of individual sugars in the standard sized sample) must be used. Therefore, the comparison was performed using the "relative concentration" values as well (Table 4). The mean Standard Deviations (0.19, and 0.15, respectively), representing the precision, are very similar to deviations found for Area % treatment, as was to be expected. The confidence ranges (95%) of the means of averages of differences between expected and observed values, viz 0.12-0.71% and 0.09-0.53%, respectively, are slightly wider than in the Area % treatment, above. In both treatments, the Calibration Averaging Routine appears to be slightly better, on an average, than the Calibration Table Routine, but the overlap

TABLE 3
STATISTICAL EVALUATION

AREA %

SPL.#		CALIBRATION TABLE			MEAN DIFFERENCES	ABS
	AVG	59.21	20.30	20.49		
	STD	0.32	0.15	0.24	0.24	
	DIF	0.79	-0.30	-0.49	0.00	0.530
2	AVG	59.82	19.97	20.21		
	STD	0.11	0.09	0.05	0.08	
	DIF	0.18	0.03	-0.21	0.00	0.139
3	AVG	59.88	19.97	20.15		
	STD	0.20	0.18	0.04	0.14	
	DIF	0.12	0.03	-0.15	0.00	0.100
1	AVG	59.47	20.40	20.12		
	STD	0.31	0.36	0.06	0.24	
	DIF	0.53	-0.40	-0.12	0.00	0.353
2	AVG	59.54	20.14	20.32		
	STD	0.32	0.42	0.37	0.37	
	DIF	0.46	-0.14	-0.32	0.00	0.307
3	AVG	59.73	20.11	20.16		
	STD	0.14	0.10	0.04	0.09	
	DIF	0.27	-0.11	-0.16	0.00	0.179
			MEAN OF ABS MEANS			0.268
			STD OF ABS MEANS			0.147
			STD ERROR OF MEAN			0.060
		CONFIDENCE RANGE (95%)			0.11 TO	0.42

SPL.#		CALIBRATION AVERAGING			MEAN DIFFERENCES	ABS
1	AVG	59.97	20.24	19.79		
	STD	0.09	0.13	0.19	0.14	
	DIF	0.03	-0.24	0.21	0.00	0.163
2	AVG	59.82	20.12	20.06		
	STD	0.36	0.29	0.09	0.25	
	DIF	0.18	-0.12	-0.06	0.00	0.123
3	AVG	60.01	20.32	19.68		
	STD	0.16	0.16	0.06	0.13	
	DIF	-0.01	-0.32	0.32	0.00	0.214
3	AVG	60.14	19.87	19.99		
	STD	0.10	0.01	0.10	0.07	
	DIF	-0.14	0.13	0.01	0.00	0.094
2	AVG	60.06	19.96	19.98		
	STD	0.18	0.21	0.03	0.14	
	DIF	-0.06	0.04	0.02	0.00	0.038
1	AVG	59.48	20.30	20.22		
	STD	0.21	0.23	0.10	0.18	
	DIF	0.52	-0.30	-0.22	0.00	0.346
			MEANS OF ABS MEANS			0.163
			STD OF ABS MEANS			0.098
			STD ERROR OF MEAN			0.040
		CONFIDENCE RANGE (95%)			0.06 TO	0.27

$T = 2.055$

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TABLE 4
STATISTICAL EVALUATION

SPL.#		AMOUNTS CALIBRATION TABLE			MEAN DIFFERENCES	ABS
	AVG	29.923	10.260	10.357		
	STD	0.349	0.037	0.083	0.156	
	DIF	0.077	-0.260	-0.357	-0.180	0.231
2	AVG	59.380	19.827	20.060		
	STD	0.399	0.219	0.206	0.275	
	DIF	0.620	0.173	-0.060	0.244	0.284
3	AVG	90.420	30.153	30.427		
	STD	0.107	0.389	0.154	0.217	
	DIF	-0.420	-0.153	-0.427	-0.333	0.333
1	AVG	30.310	10.400	10.257		
	STD	0.106	0.212	0.005	0.108	
	DIF	-0.310	-0.400	-0.257	-0.322	0.322
2	AVG	60.067	20.317	20.503		
	STD	0.451	0.341	0.512	0.435	
	DIF	-0.067	-0.317	-0.503	-0.296	0.296
3	AVG	91.450	30.793	30.860		
	STD	0.107	0.233	0.134	0.155	
	DIF	-1.450	-0.793	-0.860	-1.034	1.034
			MEAN OF ABS MEANS			0.417
			STD OF ABS MEANS			0.278
			STD ERROR OF MEAN			0.114
		CONFIDENCE RANGE (95%)			0.12 TO	0.71

SPL.#		CALIBRATION AVERAGING			MEAN DIFFERENCES	ABS
1	AVG	30.657	10.350	10.117		
	STD	0.212	0.131	0.090	0.144	
	DIF	-0.657	-0.350	-0.117	-0.374	0.374
2	AVG	60.260	20.277	20.210		
	STD	0.114	0.421	0.192	0.243	
	DIF	-0.260	-0.277	-0.210	-0.249	0.249
3	AVG	91.273	30.903	29.933		
	STD	0.143	0.321	0.167	0.211	
	DIF	-1.273	-0.903	0.067	-0.703	0.748
3	AVG	89.823	29.680	29.853		
	STD	0.192	0.094	0.260	0.182	
	DIF	0.177	0.320	0.147	0.214	0.214
2	AVG	60.207	20.013	20.030		
	STD	0.275	0.192	0.067	0.178	
	DIF	-0.207	-0.013	-0.030	-0.083	0.083
1	AVG	29.500	10.067	10.027		
	STD	0.417	0.084	0.103	0.201	
	DIF	0.500	-0.067	-0.027	0.136	0.198
			MEANS OF ABS MEANS			0.311
			STD OF ABS MEANS			0.213
			STD ERROR OF MEAN			0.087
		CONFIDENCE RANGE (95%)			0.09 TO	0.53
				T =		1.045

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does not provide the evidence for a statistically significant difference neither between the two routines, nor between the two treatments.

Similar experiment was conducted earlier using the column partly inverting sucrose (Figure 1). In this experiment, for both runs combined, average Area % values differed from expected values from 1.2% to 2.9% (T) and from 0.1% to 1.6% (AR) and Standard Deviation values varied from 0.9 to 1.7(T) and from 0.6 to 1.5(AR). Again, there is little difference in precision, measured as SD, of the two routines (1.4 and 1.1 respectively); the higher values of the Standard Deviations when compared to value obtained with newly conditioned column reflects the diminished precision for the area integration due to the bad shape of peaks under these conditions. Even under these conditions, the accuracy is marginally better when using the "AR" routine as compared to the "T" routine (grand mean difference 2.1 for T and 1.4 for AR). All results, which were generated by the use of the column not completely in calcium form were much worse than any results obtained using the column which did not invert sucrose.

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Conclusions

The Calibration Averaging Routine improved the accuracy of the results obtained using Calibration Table. However, the improvement was very small when compared to the negative effect of the sucrose inversion on the accuracy of analysis due to a gradual loss of calcium from the column. Provided that the performance of the column is monitored and properly maintained, both Calibration

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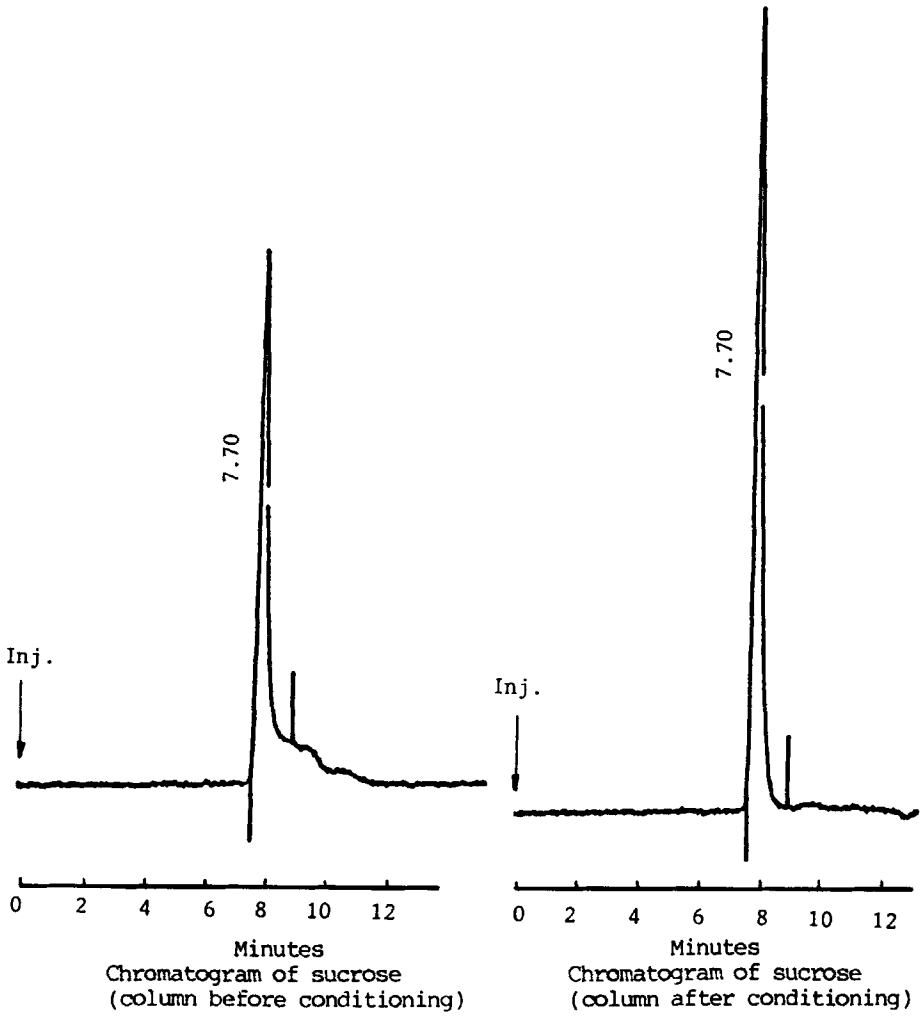

FIGURE 1

Table and Calibration Averaging Routine could be considered as an alternative to the laborious LE Method.

The Calibration Averaging Routine gives modestly better results (mean accuracy found was 0.1%) than Calibration Table (mean accuracy found was 0.3%), but is more time consuming. The programming of the Data Module initially always requires the creation of Calibration Table. The preferred use of either routine should be left to the discretion of the operator/chemist.

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