Liquid Chromatographic Analysis of Sugars, Acids, and Ethanol in Lactic Acid Vegetable Fermentations

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Routine analysis of all the major substrates and products of homolactic or heterolactic acid vegetable fermentations was made possible by the use of 2 liquid chromatographic procedures. Sucrose, glucose, fructose, and mannitol were determined on a cation exchange column in the lead form. The coefficient of variation of the added compounds (2 to 30mM) in fermented cucumber juice with salt concentrations of 0–1.5% ranged from 2.0 to 3.1%. Sucrose (1–12.5mM), malic acid, lactic acid, acetic acid, and ethanol (2–25mM) added to cucumber juice containing 0–6.0% NaCl were separated on a reverse phase column; coefficients of variation ranged between 4.3 and 5.9%. Sample preparation requires only blending and filtration prior to injection.

Liquid chromatography (LC) has been applied to many analytical problems related to foods, including measurement of sugars (1–3) and organic acids (4, 5). Marsili (6) used gas chromatography to measure glucose, fructose, and lactic acid in cucumber juice fermentations. However, analysis of the major chemical changes that may occur during vegetable fermentation requires that at least 8 compounds, including sugars, organic acids, and ethanol, be measured. Salt, which is usually added to vegetables to direct the course of fermentation (7), is a serious interference problem for the analysis of sugars by LC. It will elute from both amine-derivatized silica columns and cation exchange carbohydrate columns as a large, broad peak with retention volumes similar to sugars. Modification of chromatographic characteristics of the salt (8) or removal of salt from the samples is required for analysis of sugars in the presence of salt. This paper describes a detailed procedure for the analysis of sucrose, glucose, fructose, mannitol, malic acid, lactic acid, acetic acid, and ethanol in vegetable fermentations.

Experimental

Apparatus

(a) *Pump.*—Model 6000A (Waters Associates, Inc., Milford, MA) or equivalent.

(b) *Injector.*—Rheodyne 7125 loop injector with a 10 μL loop (Rheodyne, Inc., Cotati, CA).

(c) *Detector.*—Refractive index, Model R401 (Waters Associates) or equivalent.

(d) *Integrator.*—Spectra-Physics Model 4100 (Spectra-Physics, San Jose, CA).

(e) *Column compression module.*—RCM-100 radial compression cell (Waters Associates).

(f) *Columns.*—Aminex HPX-87P 8% cross-linked strong cation exchange resin in the lead form, 9 μm particle size, 300 × 7.8 mm, column 68°C (Bio-Rad Labs, Richmond, CA) or equivalent for sucrose, glucose, fructose, and mannitol. Radial-Pak C_{18} reverse phase, 5 μm particle size, 100 × 8 mm (Waters Associates) or equivalent for sucrose, malic acid, lactic acid, acetic acid, and ethanol.

(g) *Circulating water bath.*—Model 2067 (Forma Scientific, Marietta, OH).

(h) *Guard columns.*—For the HPX-87P, dual-guard column system was required to remove NaCl from samples and protect analytical column. Micro-guard cation exchange cartridge packed with Aminex HPX-85H and anion exchange cartridge packed with Aminex A-25 were used (Bio-Rad Labs, Richmond, CA). Guard-Pak C_{18} pre-column insert (Waters Associates) was used for C_{18} Radial-Pak column.

(i) *Filters.*—Samples were clarified with either 0.2 μm (Metricel GA-8) or 0.45 μm (Metricel GA-6) pore size, 25 mm diameter membrane placed in reusable syringe filter holder (Gelman Sciences, Inc., Ann Arbor, MI).

Reagents

(a) *Mobile phases.*—Eluant for Aminex HPX-87P column was water, distilled and passed through Millipore MilliQ water purification system (Millipore Corp., Bedford, MA). Reverse phase column was eluted with 0.05M phosphoric acid with pH adjusted to 2.5 with concentrated NH_4OH.

(b) *Standard solutions.*—Combined standard for Aminex HPX-87P column contained 10mM sucrose and 20mM each of glucose, fructose, and mannitol. Combined standard solution for reverse phase column contained 10mM sucrose and 20mM each of malic acid, lactic acid, acetic acid, and ethanol. Impurities were not detected when 50mM concentrations of standard compounds were chromatographed. In all cases, sucrose was added at half the concentration of other compounds because its response factor for refractive index detection was higher than that for other sugars and its concentration in the commodities of interest was lower than that for glucose or fructose. Sucrose, glucose, fructose, mannitol, and L-malic acid were desiccated before use. Glacial acetic acid was used without treatment. Anhydrous, nondenatured ethanol was stored over Linde 4A molecular sieve before use. Crystalline L-lactic acid was stored dessicated and frozen. (It is very hygroscopic and must be weighed quickly to prevent significant moisture uptake.) All standards were obtained from Sigma Chemical Co. (St. Louis, MO), except acetic acid, Fisher Scientific Co. (Pittsburgh, PA), and mannitol, Aldrich Chemical Co. (Milwaukee, WI).

Spiked Juice Samples

To evaluate the effect of a typical sample matrix on accuracy and precision of the analysis, the compounds, which were analyzed on the reverse phase column, were added to juice extracted from fresh cucumbers. The cucumber juice contained only a small amount of malic acid and nondetectable levels of sucrose, lactic acid, acetic acid, and ethanol. Cucumbers ('Chipper' cultivar, 4–7 cm diameter) were cut

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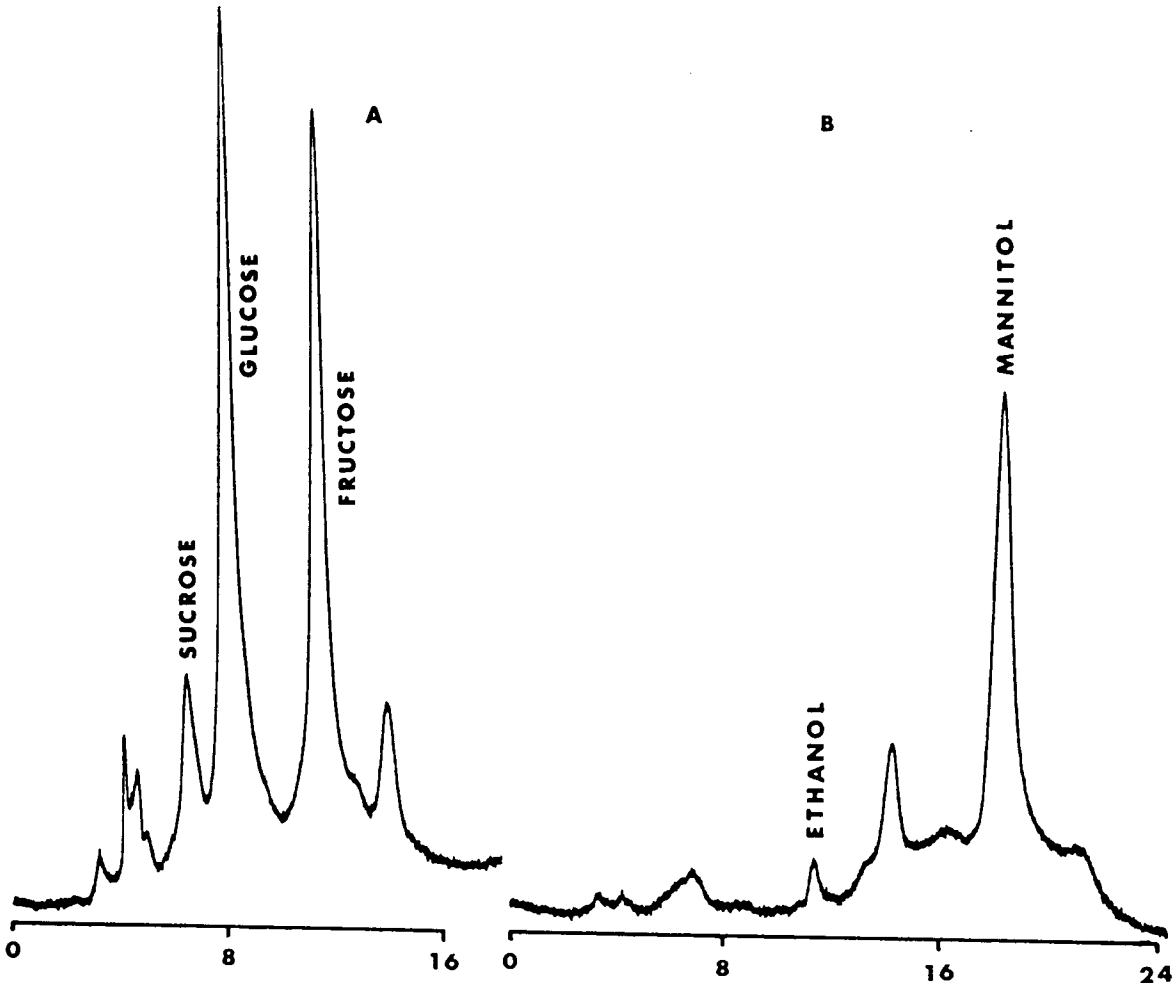

Figure 1. Sugar analysis of green bean juice before (A) and after (B) fermentation with *Lactobacillus celloblosus;* 10 μL sample was chromatographed on Bio-Rad HPX-87P column with water as eluant at 0.9 mL/min. Concentrations of analyzed compounds were: sucrose, 2.6mM; glucose, 22.0mM; fructose, 27.8mM; mannitol, 26.5mM. Other peaks on chromatograms were unidentified compounds.

into 2–3 cm pieces and frozen. The pieces were thawed and put in a wine press for juice extraction. The juice was heated to boiling to inactivate enzymes, cooled, and frozen until use. Samples were prepared by mixing 25 mL juice, which had been adjusted to pH 3.4 with HCl, with the appropriate amount of an aqueous solution of 62.5mM sucrose, 125mM each of malic acid, lactic acid, acetic acid, and ethanol. Dry NaCl and water were added to bring the final volume to 50 mL. Samples were filtered through 0.2 μm filters and frozen until use. A set of samples was prepared which contained 4.0, 20.0, and 50.0mM of each compound (sucrose concentrations were half of this) and 0, 3.0, and 6.0% NaCl.

The samples without added NaCl were chromatographed on 4 occasions over a 2-day period. One set of triplicate analyses was performed on the samples with 3.0 and 6.0% added NaCl. Samples as prepared were mixed with an equal volume of 2× concentrated elution buffer just before analysis. pH of these samples as injected into the LC column ranged from 2.27 to 2.67.

To evaluate matrix effects on sugar analysis with the HPX-87P column, sugars were added back to cucumber juice that has been fermented to remove all detectable sugars. Cucumber juice (700 mL) was filter-sterilized through a 0.2 μm sterile filter and inoculated with 1 mL of a 24-h culture of *Lactobacillus plantarum,* strain WSO, grown in MRS broth (9). Inoculated juice was incubated at 30°C. Once each day, a sample was titrated with NaOH. Sufficient 50% NaOH was added to the juice to readjust the pH to 4.5. This was continued until all detectable sugars were fermented as determined by LC. A total of 8.2 g 50% NaOH was added during fermentation. The completely fermented juice was filtered to remove bacterial cells. An aqueous stock solution of 50mM sucrose and 100mM glucose, fructose, and mannitol was prepared. Sugar stock solution was added to 25 mL fermented juice and water to prepare 50 mL solutions which contained 2, 15, and 30mM sugars (sucrose concentrations were half of this). Solutions with the same sugar concentrations were also prepared to contain 0.75 and 1.5% NaCl. These solutions were filtered through a 0.45 μm filter and frozen until use.

Samples were injected without dilution. Sugar concentrations were calculated relative to a 20mM aqueous solution of sugars (10mM sucrose). Four sets of triplicate injections of the samples without added NaCl were run over a 2-day period. Triplicate injections of the NaCl-containing samples were run once.

Preparation of Fermented Vegetable Samples

Fermented vegetables for analysis were prepared by blending samples without added liquid with a Tekmar tissue homogenizer. The homogenate was filtered through cheesecloth or centrifuged for 5 min at 2000 × g in a clinical centrifuge and then filtered by suction into a Vacutainer tube through a 0.45 μm, 25 mm filter fitted on a disposable syringe. Samples were frozen until analysis. Occasionally, fresh samples or samples collected early in a fermentation formed a precipitate after thawing. These samples were refiltered before chromatography.

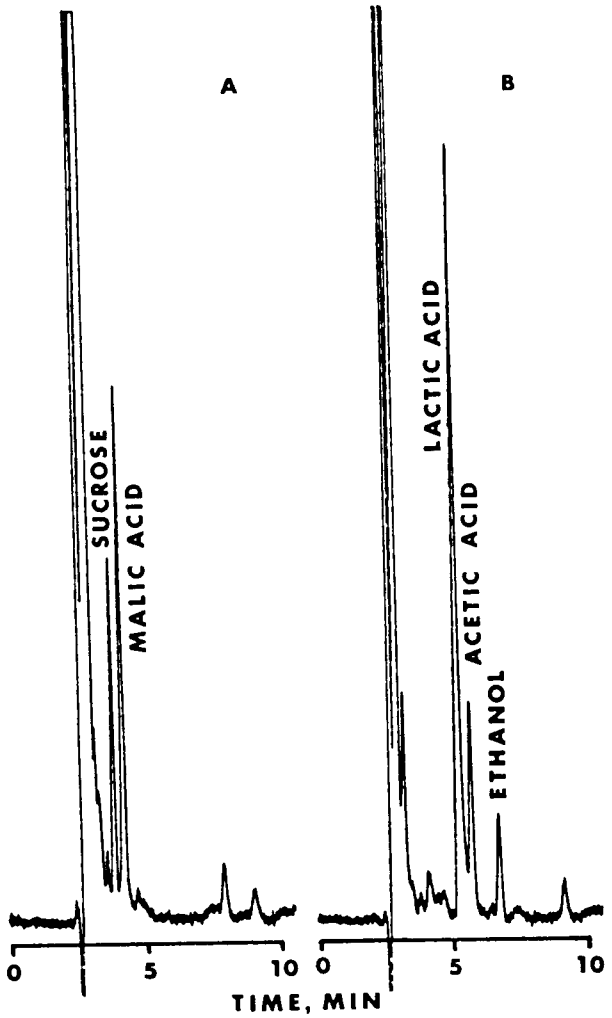

Figure 2. Sucrose, organic acid, and ethanol analysis of green bean juice before (A) and after (B) fermentation with *Lactobacillus cellobiosus*; 10 μL sample was chromatographed on Waters C_{18}, 5 μm Radial-Pak column with pH 2.5, 0.05M phosphoric acid as eluant at 1.0 mL/min. Concentrations of compounds were: sucrose, 2.6mM; malic acid, 9.3mM; lactic acid, 45.1mM; acetic acid, 20.3mM; ethanol, 21.7mM. Other peaks on chromatograms were not identified.

Brine samples were filtered through a 0.45 μm pore size filter directly into vacuum tubes and frozen. When brines were cloudy with suspended bacteria, the rate of filtration was improved by centrifugation of the sample for 5 min at 2000 × g to remove the bacterial cells.

Chromatographic Procedure

Sugar analysis on the HPX-87P column was done with the column in a water bath at 68°C with the guard columns at room temperature to prevent sucrose hydrolysis. A 10 μL sample was injected onto the column with a loop injector. Water was pumped through the column at 0.9 mL/min. Sugar concentrations were calculated by comparison to the peak height of 20mM standards (10mM for sucrose). If the samples contained NaCl, they were diluted with water so the salt concentration in the injected sample was 1.5% or less.

Samples for analysis of acids, ethanol, and sucrose on the C_{18} Radial-Pak column were mixed with an equal volume of 0.1M, pH 2.5 phosphate buffer before injection onto the column. This is done to reduce baseline upset at the solvent front and to protonate the organic acids. The flow rate was 1.0 mL/min. Sample component concentrations were calculated by comparison of the peak height with 20mM external standards (10mM sucrose).

For all analyses, the refractive index detector was set at an attenuation of 8× and 100 mV output. The SP4100 integrator was set for a 100 mV input. Peak height measurements were made with the integrator.

Results and Discussion

Chromatography of Sugars with Cation Exchange Column

Figures 1 and 2 show typical separations obtained from samples of fresh and fermented green beans, prepared by blending, filtration, and, in certain instances, dilution before injection into the chromatograph. Figure 1A shows the results of chromatography on the HPX-87P column. Sucrose, glucose, and fructose in the freshly brined beans were well separated. After fermentation (Figure 1B), mannitol was the major compound on the chromatogram. On this column, ethanol also eluted with a retention time almost identical to fructose. When both fructose and ethanol are present in a sample, the ethanol can be measured on the reverse phase column. The ethanol contribution can then be subtracted from the fructose peak on the HPX-87P column to estimate fructose concentration. Use of a column similar to the HPX-87P in the calcium instead of the lead form changes the relative retention of these compounds and may eliminate this problem.

The amount of NaCl in samples injected into the HPX-87P must be monitored because the guard columns have a limited exchange capacity. Approximately fifty 10 μL fermented vegetable sample injections, each with 1.25% NaCl, could be chromatographed before a visible salt peak would occur on the column. The guard columns can be used until the eluted NaCl interferes with a component of interest. When the component concentrates with high enough, samples were diluted to contain less than 1.5% NaCl before injection. Even at 1.5% NaCl, some baseline upset occurred which interfered with analysis of fructose.

New anion exchange guard columns typically will partially bind sugars. In one case, the peak heights of a standard solution of sugars in a new column were reduced by 13% for glucose, 14% for fructose and mannitol, and 22% for sucrose, relative to a conditioned guard column. A new guard column may be conditioned by pumping 68°C water through it for at least 5 h. For convenience, columns were often left in the water bath overnight before use. We do not know the mechanism by which heating the guard column eliminates sugar binding. As described above, guard columns must be kept at room temperature when analyses are performed.

Chromatography of aqueous solutions of glucose, fructose, and mannitol on the HPX-87P column resulted in a linear relationship between peak height and sugar concentrations over a 1–100mM concentration range. Table 1 shows results of the analysis of these compounds over a 2–30mM concentration range and sucrose over a 1–15mM range in fermented cucumber juice at each of 3 salt concentrations. The slope, intercept, coefficient of variation of the linear regression, and coefficient of determination for the relationship between added and analyzed sugar were calculated at each salt concentration. Overall values were those pooled over the 3 salt concentrations and calculated. The coefficients of variation at the 2mM concentration (1mM sucrose), pooled over the 3 salt concentrations, gave an estimate of variability at low sugar concentrations. Coefficients of determination (R^2) were 0.999 or higher for each compound at each salt concentration, indicating an excellent linear relationship between added and measured sugar concentrations in the fermented cucumber juice matrix. An ideal analysis should result in a linear rela-

Table 1. Regression analysis of analyzed vs added sucrose, glucose, fructose, and mannitol in completely fermented cucumber juice with 0, 0.75, and 1.5% NaCl added

Compound	NaCl concn, %	Slope[a]	Intercept[b]	CV over 2–30mM range, %	CV for 2mM concn, %
Sucrose	0	1.00	0.06	2.2[d]	
	0.75	1.03**	0.17*	1.4	
	1.5	1.03**	0.13*	1.1	
	Overall[e]	1.01	0.09	3.1	6.8[f]
Glucose	0	0.99**	0.25**	1.5	
	0.75	1.02	0.44	2.4	
	1.5	1.01	0.44*	1.9	
	Overall	1.00	0.31*	2.6	9.2
Fructose[c]	0	0.99	0.21**	1.5	
	0.75	1.01	0.43*	1.5	
	1.5	—	—	—	
	Overall	1.00	0.25*	2.0	8.4
Mannitol	0	0.99	0.11	1.8	
	0.75	1.02**	0.23	1.2	
	1.5	1.01	0.40	2.0	
	Overall	1.00	0.18	2.6	8.8

[a]Asterisks indicate that slope is statistically significantly different from expected 1.00.
[b]Asterisks indicate that intercept is statistically significantly different from 0.
[c]Fructose could not be accurately measured because of baseline upset at 1.5% NaCl.
[d]Sucrose concentration ranged from 1 to 15mM. Coefficient of variation is based on standard deviation of regression model.
[e]Calculated by pooling all data over 3 NaCl concentrations.
[f]Lowest sucrose concentration was 1.0mM.

tionship between added and analyzed compounds with a slope of 1.0 and an intercept of 0. Table 1 shows that the slopes were near 1.0 and the intercepts were near 0 for each compound analyzed. The high precision of the analysis resulted in statistically significant deviations of the slopes from 1.0 ($P \leq 0.01$) at some NaCl concentrations tested. However, when the regression was pooled over the three NaCl concentrations used, the overall slopes were not significantly different from 1.0. The intercept was significantly different from the expected zero in several cases, but the deviations were too small to be of practical concern in the analysis. The coefficients of variation for the 4 compounds analyzed over a 2-day period ranged from 2.0 to 3.1% when the regression was pooled over the 3 salt concentrations. The coefficients of variation for the lowest component concentrations analyzed ranged from 6.8 to 9.2%. Baseline upsets, which occurred when 1.5% NaCl was added to the samples, prevented the accurate analysis of fructose.

Chromatography of Sucrose, Acids, and Ethanol on Reverse Phase Column

Separation of compounds on the reverse phase column before and after fermentation is shown in Figure 2. Monosaccharides, salts, and other polar compounds elute at the solvent front and cause no interference with the separation of components of interest. Since sucrose separates on the C_{18} column, it can be measured on both of the columns used for analysis of fermentation components. Sucrose, malic acid, lactic acid, acetic acid, and ethanol can all be separated throughout the course of fermentations. The 2 LC procedures described here allow analysis of all major fermentation substrates normally formed in vegetable fermentations, except CO_2. The procedure for ethanol analysis does not work well with alcoholic fermentations, such as wine fermentations, because the large ethanol peak will interfere with analysis of minor acid components. This procedure can also be used to measure other organic acids, such as citric acid, succinic and fumaric acid, that elute after ethanol (5), although these compounds were not evaluated in these experiments.

One problem with the use of C_{18} reverse phase chromatography is that the retention times and resolution of the components gradually decline. This remains a general problem with this type of column packing material. In many cases, the useful life of a column can be extended by increasing the polarity of the elution solvent. This is not possible with an aqueous buffer as the eluant. By decreasing the temperature of the column to 12°C, when it began to lose resolution, useful column life can be extended significantly. This was done with Radial-Pak columns by placing the radial compression module in a double-walled plastic bag to protect it from water and submerging the whole module in a water bath. Adjustments must be made on the module to account for contraction and expansion of the hydraulic fluid in the compression cell during cooling and warming.

Table 2 shows the results of chromatography of samples with 0, 3, and 6% NaCl. The results were calculated at each salt concentration and then over all 3 salt concentrations, as was done for the sugar analysis. The coefficients of determination ranged from 0.995 to 0.999. Since the sucrose curve was linear with an overall slope of 1.0 for both columns, this indicated that it was not hydrolyzed under the acid conditions normally encountered in samples of fermented vegetables. Malic acid had a slope less than 1.0, and the lactic slope was greater than 1.0. We do not know the reason for this deviation from the expected slope for these compounds. Malic acid had a nonzero intercept because 3.8 mM malic acid was present in the starting cucumber juice. The other deviations of the intercepts were not large enough to be of practical consequence in the analysis. The coefficients of variation ranged from 4.3 to 5.9% when results were calculated over 3 component concentrations and 3 salt concentrations during a 2-day period. At the lowest concentration of the compounds analyzed, the coefficients of variation ranged from 2.7 to 7.7%.

The application of these 2 LC procedures has made it practical to routinely analyze all major substrates and products, with the exception of CO_2, that commonly occur in vegetable fermentations. This allows carbon balances to be calculated for complex heterolactic acid fermentations (10) and makes it possible to follow the course of lactic acid fermentations in detail (11).

We have recently purchased a column with an anion exchange packing material similar to the Bio-Rad HPX-87P column, except that the counter ion is calcium rather than lead. The column provided reproducibility similar to the lead

Table 2. Regression analysis of analyzed vs added sucrose, malic acid, lactic acid, acetic acid, and ethanol in cucumber juice with 0, 3, and 6% NaCl added

Compound	NaCl concn, %	Slope[a]	Intercept[b]	CV over 2–25mM range, %	CV for 2mM concn, %
Sucrose	0	0.99	0.23	4.0[c]	
	3.0	0.99	0.18	6.2	
	6.0	0.99	0.22	4.8	
	Overall[d]	1.00	−0.04	5.9	4.9[e]
Malic acid	0	0.99	3.59**	2.2	
	3.0	0.93*	3.83**	6.9	
	6.0	0.93*	3.99**	5.7	
	Overall	0.95*	3.80**	5.6	2.7
Lactic acid	0	1.09**	−0.37**	3.8	
	3.0	1.06	−0.32	8.6	
	6.0	1.07	−0.29	4.6	
	Overall	1.08**	−0.33**	5.9	4.3
Acetic acid	0	0.99	0.23	4.3	
	3.0	0.99	0.18	4.8	
	6.0	0.99	0.27	4.4	
	Overall	0.99	0.21*	4.3	6.3
Ethanol	0	0.99	0.19	3.0	
	3.0	0.98	0.22	7.6	
	6.0	0.99	0.28*	3.0	
	Overall	0.99	0.23*	4.8	7.7

[a]Asterisks indicate that slope is significantly different from expected 1.00 at 0.05 (*) or 0.01 (**) level.
[b]Asterisks indicate that intercept is significantly different from 0 at 0.05 (*) or 0.01 (**) level.
[c]Sucrose concentration ranged from 1 to 12.5mM. Coefficient of variation is based on standard deviation of regression model.
[d]Calculated by pooling all data over 3 NaCl concentrations.
[e]Lowest sucrose concentration was 1.0mM.

column, but with two advantages: Small amounts of NaCl did not interfere with sugar analysis because salt eluted from the column before the sugars, and interference with fructose did not occur because ethanol was separated from the other compounds.

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