Malic Acid Analysis in Cucumber Juice and Fermentation Brines in the Presence of Interfering Fructose

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ABSTRACT

A procedure was developed for HPLC analysis of malic acid in the presence of an interfering fructose peak with an Aminex HPX-87H column. Fructose in cucumber juice or fermented cucumber brine was reduced to mannitol and sorbitol with sodium borohydride. The sugar alcohols eluted after malic acid and did not absorb light at 210 nm so that malic acid could be quantitatively determined either by refractive index or ultraviolet detectors. Lactic acid, acetic acid, and ethanol could also be determined in the sample after reduction of fructose.

Key Words: malic acid, cucumbers, fermentation, fructose, lactic acid

INTRODUCTION

MALIC ACID analysis in cucumber extracts and fermentation brines has been done by reversed phase HPLC (McFeeters et al., 1984). However, due to relatively rapid loss of resolution of malic acid from lactic acid on reversed phase columns, analysis on a polystyrene cation ion exchange column is preferred. Retention times of sugars and organic acids, including malic acid, are stable for many samples on this type column. Malic acid, the major organic acid in pickling cucumbers (McFeeters et al., 1982), is not completely resolved from fructose with the usual concentrations of sulfuric acid (≈ 0.01N) that resolve the other components of interest. Since the refractive index (RI) detector response for fructose is similar to that of malic acid, the malic acid peak may be obscured by a large fructose peak. Detection by ultraviolet (UV) absorption at 210 nm would be better, but fructose absorbs light to a small degree at that wavelength such that a large amount of fructose in a sample could prevent quantification of lower concentrations of malic acid. This is a common situation in analysis of fruit and vegetable products.

The same problem occurs in wines. Frayne (1986) obtained resolution of malic acid from fructose by using two Aminex HPX-87H columns in series but the method required longer analysis times and an additional column. Schneider et al. (1987) reported resolution with 0.0026N H_2SO_4 as the eluant, but 0.013N H_2SO_4 gave better resolution of other wine components. For cucumber fermentation brines, Lazaro et al. (1989) circumvented this resolution and detection problem by use of RI and UV detectors in series, and a set of simultaneous equations which used peak height values from both detectors to quantitate malic acid and fructose from incompletely resolved peaks. Our objective was to demonstrate the analysis of malic acid, lactic acid, acetic acid, and ethanol in cucumber juice and fermentation brines after reduction of interfering fructose to mannitol and sorbitol with sodium borohydride. Reduction would convert fructose to sugar alcohols which have a slightly longer retention time than fructose and no UV absorption. The result would be no interference in detection of malic acid by UV absorption. In addition, there was sufficient resolution of the malic acid from sugar alcohols such that it could also be quantitated with the RI detector. Sodium borohydride also reduces glucose to sorbitol.

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MATERIALS & METHODS

HPLC was performed with an Aminex HPX-87H column (7.8 × 300 mm) with a cation guard column (#125-0129, Bio-Rad Laboratories, Richmond, CA). The eluant solution was 0.01N H_2SO_4 at a flow rate of 0.8 mL/min. The chromatograph consisted of a Waters 6000 pump (Millipore Corp., Milford, MA); a 7125 injector with a 10 μL loop (Rheodyne Inc., Cotati, CA); a Waters column heater set at 65°C; a Varichrom variable-wavelength, visible/UV detector (Varian Instruments Inc., Palo Alto, CA) set at 210 nm; and a Waters, model 410, RI detector connected in series. Data were collected on two channels of a chrom-1AT A/D converter board and LabCalc chromatography software (Galactic Industries Corp., Salem, NH) installed on a Gateway 2000 (Sioux City, SD), model 486/25, personal computer. Peaks were analyzed using peak heights and external standards prepared in water.

Cucumber juice was prepared from commercial pickling cucumbers 38-44 mm in diameter (Daeschel et al., 1988) and frozen until use. Cucumber fermentation brine was prepared by fermentation of cucumbers with *Lactobacillus plantarum*. Cucumbers (38–44 mm in diameter) were covered with an equal volume of brine, which contained 106 mM acetic acid and 36 mM calcium hydroxide to give an initial pH of 4.6. No salt was added during fermentation. Jars were inoculated with 10^6 CFU/mL *L. plantarum* MOP3 from the laboratory culture collection. After fermentation, 4% NaCl was added to give a fermentation brine with NaCl concentration similar to that expected in pickel samples.

Solutions were prepared to contain known amounts of the compounds of interest added to cucumber juice or fermented cucumber brines with 4% NaCl and no detectable glucose or fructose. Malic acid, lactic acid, acetic acid, and ethanol were added to the cucumber juice in the range of 1 to 50 mM. Since there was a two-

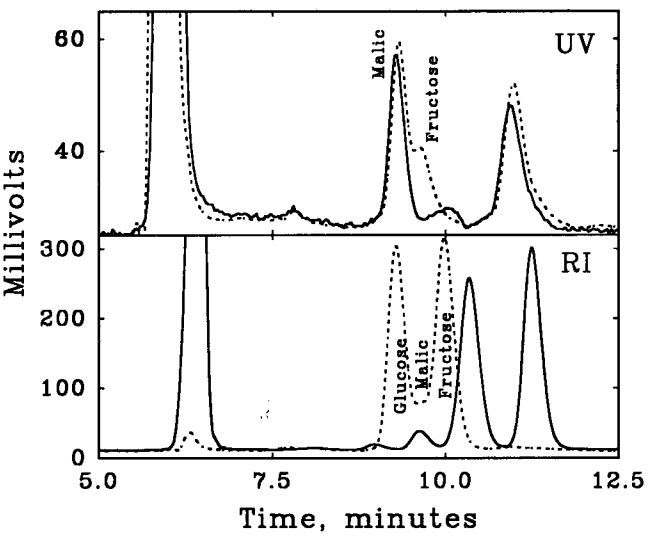

Fig. 1—Effect of sodium borohydride reduction of cucumber juice samples on the removal of interference from the malic acid peak by glucose and fructose. UV = ultraviolet detection at 210 nm; RI = refractive index detection. ----- before borohydride reduction; ——— after borohydride reduction.

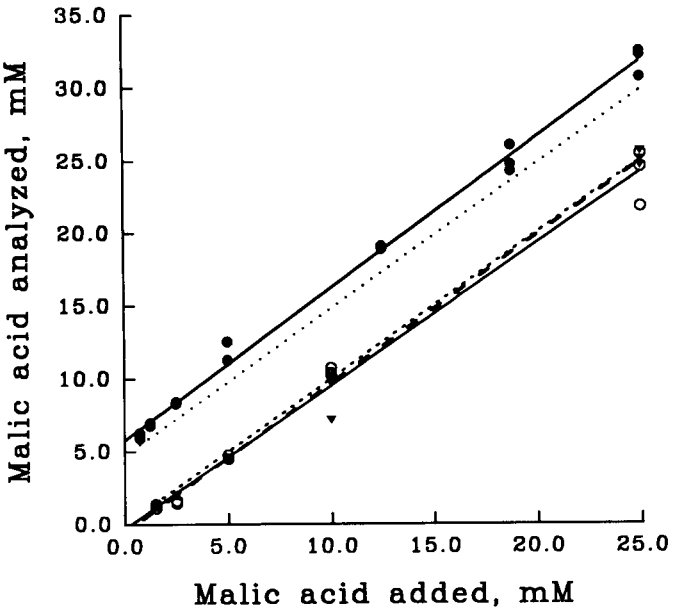

Fig. 2—Determination of malic acid by UV absorption at 210 nm in cucumber juice and fermented cucumber brine with 4% NaCl after sodium borohydride reduction of fructose and glucose. . . . . expected malic acid concentration; ▼ - - - ▼ malic acid in fermentation brine with 10 mM added fructose and glucose; ○ - ○ malic acid in fermentation brine with 50 mM added fructose and glucose; ● - ● malic acid in cucumber juice.

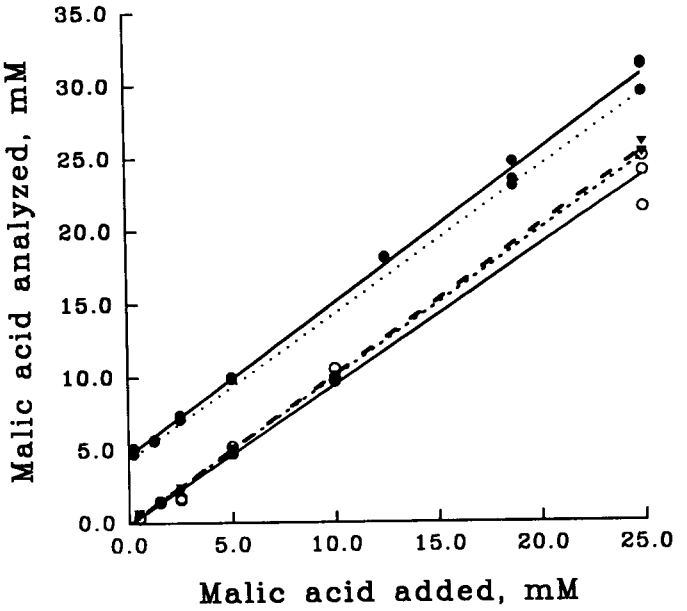

Fig. 3—Determination of malic acid by RI detection in cucumber juice and fermented cucumber brine with 4% NcCl after sodium borohydride reduction of fructose and glucose. . . . . expected malic acid concentration; ▼ - ▼ malic acid in fermentation brine with 10 mM added fructose and glucose; ○ - ○ malic acid in fermentation brine with 50 mM added fructose and glucose; ● - ● malic acid in cucumber juice.

fold dilution of samples during the borohydride reduction, the concentration range as analyzed was 0.5 to 25 mM. The juice contained ≈ 50 mM each of glucose and fructose after a 1:1 dilution resulting from addition of the compounds and water. The fermentation brine was also diluted 1:1 as a result of addition of glucose, fructose, malic acid, and ethanol. Two sets of solutions were prepared. Glucose and fructose (10 mM) were added to one set and 50 mM of sugars were added to the second set. Malic acid and ethanol were added in the range of 1 to 50 mM. Since the brine contained high levels of lactic and acetic acids, those compounds were not added.

Reduction of fructose and glucose was accomplished by preparing stock solutions of 285 mg $NaBH_4$ in 5.0 mL 0.25N NaOH, 1 mg/mL pronase (Sigma Chemical Co., St. Louis, MO) in water, and 7N H_2SO_4. A small hole was made with a hot, 27-gauge needle in the top of 1.5 mL, disposable plastic centrifuge tubes. Pronase (75 μL) was added to 200 μL of sample and then incubated for 2 hr at 40°C. Pronase treatment reduced foaming, particularly for cucumber juice samples. $NaBH_4$ solution (100 μL) was added to reduce sugars. Vigorous bubbling occurred due to release of hydrogen gas. The sample was again incubated for 2 hr at 40°C to assure complete reduction. To decompose remaining sodium borohydride, 25μL 7N H_2SO_4 was carefully added. Rapid release of

Table 1—Regression analysis of added versus analyzed concentrations of compounds in cucumber juice detected by UV absorption at 210 nm and by refractive index

Compound	Detector	Slope	Standard deviation of the slope	Intercept	Standard deviation of the intercept	r^2
Malic acid	UV	1.0449[a]	0.0121	4.5647	0.1458	0.9971
Malic acid	RI	1.0530	0.0154	5.5353	0.1865	0.9953
Ethanol	RI	0.7809	0.0643	1.9385	1.0937	0.9365
Lactic acid	UV	1.0623	0.0138	−0.1929	0.1924	0.9973
Lactic acid	RI	1.1234	0.0255	−0.4145	0.3886	0.9934
Acetic acid	UV	1.2032	0.0385	−1.1768	0.6551	0.9899
Acetic acid	RI	1.0383	0.0319	0.7954	0.4874	0.9878

[a] A slope for the regressions of 1.0 shows that the concentrations analyzed were equal to the added concentrations.

Table 2—Regression analysis of added versus analyzed concentrations of compounds in fermented cucumber brine detected by UV absorption at 210 nm and by refractive index

Sugars added to brine, mM	Compound	Detector	Slope	Standard deviation of the slope	Intercept	Standard deviation of the intercept	r^2
10	Malic acid	UV	1.0194[a]	0.0074	−0.0719	0.0828	0.9992
50	Malic acid	UV	0.9518	0.0218	−0.0768	0.2446	0.9917
10	Malic acid	RI	1.0092	0.0171	−0.3891	0.1779	0.9946
50	Malic acid	RI	0.9784	0.0219	−0.2831	0.2337	0.9911
10	Ethanol	RI	0.7993	0.0150	5.6002	0.1567	0.9933
50	Ethanol	RI	0.8153	0.0240	6.4600	0.2557	0.9847

[a] A slope for the regressions of 1.0 shows that the concentrations analyzed were equal to the added concentrations.

hydrogen again occurred. When bubbling stopped, tubes were centrifuged and the supernatant sample solution was injected. After addition of sulfuric acid, samples could be frozen until used. Reduction reactions were done in triplicate and analyzed for each level of malic acid added.

RESULTS & DISCUSSION

OVERLAID chromatograms with RI and UV detectors of cucumber juice before and after reduction with sodium borohydride (Fig. 1) showed that before reduction, the malic acid peak was completely obscured by glucose and fructose peaks in the RI chromatogram. After reduction, there was some tailing of the sugar alcohol peak into the malic acid peak, but the peak could be readily quantitated. In the UV/visible chromatogram, the malic acid peak was clearly visible, but there was substantial interference from the slight UV absorption by the large quantity of fructose present. Since reduction of fructose to sugar alcohols completely eliminated UV absorption in addition to shifting elution to a slightly longer time, no interference with malic acid remained. Complete conversion of both glucose and fructose to sugar alcohols by this reduction procedure was verified. We ran chromatograms of samples of sodium borohydride-treated cucumber juice and fermentation brine with up to 100 mM added sugars on a sugar analysis column (McFeeters et al., (1984). No trace of either glucose or fructose was observed, and the appropriate sugar alcohol peaks were present.

The relationship between added and analyzed malic acid with a UV/visible detector at 210 nm (Fig. 2) showed the analyzed malic acid was equal to the added malic acid, whether 10 mM or 50 mM glucose and fructose had been added to fermented cucumber brine with 4% NaCl. There was a linear relationship between the added and analyzed malic acid in cucumber juice samples as well. However, in the cucumber juice matrix the slope of the line was slightly greater than the expected value of 1.0 (Table 1). The intercept greater than zero is due to the malic acid naturally occurring in cucumber juice.

Analysis of malic acid in the same sets of samples with a RI detector gave essentially identical results (Fig. 3), provided the peak height was measured from the top of the peak to the chromatogram baseline. Calculation of the height of the malic acid peak using a shoulder-skimming integration algorithm resulted in under-estimation of the concentration. Due to interference from the leading edge of the large sugar alcohol peak which was present in the RI chromatograms (Fig. 1), 0.75 mM added malic acid could not be detected, but 1.5 mM malic acid was detected. With the UV detector where there was no response to sugar alcohols, the lowest level malic acid (0.5 mM) could be detected.

A linear response resulted from both detectors to the lactic and acetic acids added to cucumber juice (Tables 1 and 2). However, the slope of the lactic acid response was slightly greater than 1.0 with both detectors. There was a small amount of interference by the tailing edge of the sugar alcohol peak in the lactic acid peak with the RI detector. Acetic acid had a slope that was not different from 1.0 within experiemental error with the RI detector. However, the slope was 1.21 for the UV/visible detector. An unidentified peak (retention time 12.6 min) interfered with the acetic acid peak (retention time 13.2 min), but the reason for a slope so much greater than 1.0 is not known.

Ethanol could only be detected by the RI detector. In both cucumber juice and fermentation brines there was a linear response to the amount of ethanol added. However, the slope was only 0.8 for all three sets of samples analyzed. Since ethanol is volatile, possibly some loss was caused by evaporation during the sugar reduction reaction, particularly when vigorous release of hydrogen gas occurred. Intercepts greater than zero for the brine samples were due to the presence of a small amount of ethanol in the fermentation brine.

This method differs from previous approaches in that it does not rely upon manipulating the difficult fructose/malic acid resolution. Unlike Lazaro et al. (1989), two detectors were not required. Either the UV or RI detector was sufficient. This is the only method which allows analysis of malic acid on a RI detector alone when fructose is present. Sugar determinations must be done in a separate analysis. However, analysis of other compounds of interest in fermented samples may be done on the reduced samples.

CONCLUSIONS

SODIUM BOROHYDRIDE reduction of fructose and glucose allowed analysis of malic acid by either UV or RI detectors in cucumber juice and fermented brines containing NaCl. Other compounds of interest in the cucumber fermentation could also be analyzed. However, the sample matrix appeared to affect analysis in some cases such that preparation of standards in the matrix of interest may be required for best accuracy.

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