Determination of Organic Acids During the Fermentation and Cold Storage of Yogurt¹

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ABSTRACT

The objective of the present study was the separation and quantification of orotic, citric, pyruvic, lactic, uric, formic, acetic, propionic, butyric, and hippuric acids in a single isocratic analysis by HPLC. Two methods of extraction were compared: 1) acetonitrile and water and 2) 0.01 N H₂SO₄. Recoveries of orotic, lactic, acetic, and propionic acids were 90% for both methods. Recoveries of citric, pyruvic, uric, butyric, and hippuric acids were not satisfactory with the acetonitrile method, but were acceptable using the H₂SO₄ extraction procedure. Yogurts were manufactured under laboratory-scale conditions, and samples were analyzed during fermentation and after storage at 4°C. Samples were analyzed for pH and organic acids. All of the organic acids exhibited varying degrees of increases and decreases during fermentation and storage. Formic and butyric acids were not detected under the conditions of this study.

(Key words: high performance liquid chromatography, organic acids, fermentation, yogurt)

INTRODUCTION

Organic acids are particularly important for the final properties of processed foods such as fermented dairy products. Organic acids are significant as natural preservatives and for sensory characteristics of the product. Some organic acids have been implicated as possible factors in the cure and prevention of certain diseases; lactic acid has been related to the inhibition of certain pathogenic bacteria in yogurt (11). Orotic acid is a growth factor for lactobacilli (14) and has been identified as a possible factor in milk that could help reduce the incidence of cholesterolemia in humans (10).

The advantages of using HPLC in the determination of organic acids are speed, simultaneous analysis of several acids, and easy sample preparation. An ion-exchange separation method has been described for the organic acids that are present in different dairy products (8). Difficulties were encountered in resolving uric and formic acids in a single analysis, which made it necessary to run the sample at two different temperatures to quantify both acids. More recently, the same basic methodology has been used to determine organic acids and carbohydrates in Cheddar cheese (4). In that study, orotic and citric acids coeluted and could only be quantified in two separate analyses using their different absorbance characteristics at 280 and 214 nm. Others have studied organic acids in buttermilk (6), cheese (7), and yogurt from milk concentrated by ultrafiltration (3). The objective of our study was to attain, in a single run, the separation and quantitation of 10 organic acids: orotic, citric, pyruvic, lactic, uric, formic, acetic, propionic, butyric, and hippuric acids. Two methods of extraction of the organic acids from yogurt were compared, and the methodology was applied to the study of
the evolution of organic acids during the fermentation and cold storage of plain yogurt.

MATERIALS AND METHODS

Extraction of Organic Acids

The two methods for extraction of the organic acids from yogurt were 1) acetonitrile and water: 5 ml of water were added to 4 g of yogurt and diluted to 25 ml with acetonitrile (8), and 2) diluted H$_2$SO$_4$: 4 g of yogurt were diluted to 25 ml with .01N H$_2$SO$_4$. A concentration of .013N H$_2$SO$_4$ was necessary for nonfermented milk samples. Extracts were filtered through .2-pm Titan filters (Science Resources, Inc., North Brunswick, NJ). Extractions were carried out at least in duplicate.

Recovery of Organic Acids

Individual organic acid solutions were prepared in water except those of uric and orotic, which were prepared in .01N NaOH. The recovery was in duplicate by mixing 1 and 2 ml of a concentrated standard solution to 4 g of yogurt and extraction of the organic acids following the two described procedures. Real concentrations of acids were calculated taking into account the purity of the standards.

HPLC of Organic Acids

The HPLC method was a modification of one previously described (8) and was carried out using a Waters system (Waters Associates, Milford, MA) consisting of a 501 pump, 715 Wisp Auto sampler, 410 UV-Visible Detector, SIM interface, and Baseline/Maxima software for control of pump and data collection and handling, installed in a NRC Powermate Computer with NEC Printer (NEC Technologies, Inc., Boxborough, MA). Separation was performed with an AMINEX HPX-87H ion-exchange column (300 x 7.8 mm) and guard column with disposable cartridges H$^+$ (Bio-Rad Labs, Richmond, CA), preceded by a precolumn with disposable 2-μm filters. All three elements were heated at 65 to 66°C with a Bio-Rad HPLC column heater. The mobile phase was prepared by dissolving H$_2$SO$_4$ from Titrisol ampules (Merck, Darmstadt, Germany) in HPLC grade water (Ultrapure water system; Barnstead, Boston, MA) and was filtered through 45-μm filters by means of a filtering device (Laser Research Lab, Los Angeles, CA). After many assays under different conditions, the concentration of .0075N H$_2$SO$_4$ in the mobile phase, a flow rate of .7 ml/min, and detection at 210-nm wavelength were used. An injection volume of 10 μl was used for standards and samples. Quantification of organic acids was carried out by the external standard method. Linear regression curves (R$^2$ > .9999) based on peak height were calculated for the individual organic acids after duplicate injections of five aqueous standard solutions (stock solutions; Sigma Chemical Co., St. Louis, MO) covering a broad range of concentrations. Uric and orotic acids were dissolved in .01N NaOH. The standards were very stable under refrigeration. Three standard solutions were injected each day to verify the reproducibility of the method. Identifications were based on matching retention times of standards.

Yogurt Manufacture

Yogurts were manufactured from reconstituted skim milk and pasteurized homogenized whole milk standardized to 12% SNF and 1% fat. The mix was heated at 85°C for 30 min, cooled to 42°C, inoculated with .04% of a concentrated frozen starter culture [consisting of Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus salivarius ssp. thermophilus, (1:1) (CH-3; Chr Hansen’s Lab, Inc., Milwaukee, WI], and incubated at 42°C to a final pH of 4.4 (7 to 8 h). The coagulum was then broken, and the stirred yogurt was aseptically transferred to plastic cups and stored at 4°C for 4 wk. Duplicate trials were conducted.

Sampling for Analysis

Samples were taken under sterile conditions in Whirl-Pak (Fort Atkinson, WI) sampling bags at 1-h intervals during fermentation and after 4 wk of storage at 4°C. The pH was measured with a pH meter (SA250; Orion Research Inc., Boston, MA).

RESULTS AND DISCUSSION

HPLC Analysis of Organic Acids

Several combinations of H$_2$SO$_4$ concentration in the mobile phase, temperature, and flow rate were evaluated. Conditions leading to the
enhanced resolution of uric and formic acids caused a reduction in the resolution of lactic and uric or of orotic and citric acids. The optimal conditions determined in our study were 0.0075N H₂SO₄ mobile phase; temperature of 65 to 66°C applied to precolumn, guard column, and analytical column; and flow rate of 0.7 ml/min. All organic acids could be identified and quantified under these conditions. Detection at 210 nm was preferred because most acids gave the highest response at this wavelength. Figure 1 shows an HPLC chromatogram of a standard solution of 10 organic acids that were eluted in 30 min. The reproducibility of the method was very good. The coefficient of variation between concentrations in duplicate extractions was <2%. Variation in retention times of organic acids was insignificant, except when the guard column lost its ability to bond cations. Because of the Ca²⁺ concentration of the extracts, the cartridge had to be replaced every 40 to 50 injections. The column also needed to be cleaned and regenerated (following the manufacturer’s instructions) to maintain efficiency.

Recovery of Organic Acids

Table 1 shows the recovery of organic acids for the two extraction procedures. Two different levels of standard addition were assayed for both methods. The concentration of organic acids in the original yogurt varied depending on the extraction method; citric acid was dramatically affected. Concentration of this acid (2.1 mg/g) was found within the upper levels reported in milk (1, 15) when the H₂SO₄ procedure was used. The recovery of citric acid concentrations was much lower (.65 mg/g) with the acetonitrile-water extraction method and similar to those reported by others (3, 6, 8) using the same extraction procedure. In milk, most citrate is bound to Ca, forming relatively soluble complexes (mainly the anions Ca Cit⁻ and Mg Cit⁻). Another part is as free ion (Cit³⁻, H₂Cit⁻, H₂Cit⁻) species. A small amount of citrate is also associated with casein micelles (5). Under the conditions of our study, most citrate precipitated with the caseins when acetonitrile was added. This method limited us to only the extraction of the soluble portion, but that portion was completely dissolved and recovered under aqueous acid conditions. Experiments in our laboratory showed that, at low pH (by addition of H₂SO₄ and 40% acetonitrile, a second precipitate (after filtration through .2 μm) was formed. This precipitate, once removed, dissolved, and analyzed by HPLC, consisted basically of citrate.

Good recoveries of orotic, lactic, acetic, and propionic acids were obtained with both methods (>90%). However, recoveries of citric, pyruvic, uric, butyric, and hippuric acids were not satisfactory with the acetonitrile method. For this reason, we decided to use the .01N H₂SO₄ extraction procedure to study the fermentation of yogurt.

Organic Acids During Fermentation and Storage of Yogurt

Figure 2 shows a typical HPLC chromatogram of a fresh yogurt. Orotic, citric, pyruvic, lactic, uric, acetic, and hippuric acids were easily identified and quantified in milk and yogurt samples. Butyric acid was not detected under the conditions used. The detection of formic acid in milk or in yogurt was difficult because of the presence of an unidentified peak (peak g in Figure 2). The elution time of peak g was between uric and formic acids. If the organic acid extract was maintained at 21°C for several hours, this unknown peak increased, and uric acid decreased. This effect
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¹Formic acid plus the unknown peak labeled g.
Figure 2. An HPLC chromatogram at 210 nm of a .01N H2SO4 extract of fresh plain yogurt. Organic acids: 1) orotic, 2) citric, 3) pyruvic, 4) lactic, 5) uric, 7) acetic, and 8) propionic; a) phosphates and unretained compounds; c) glucose; d) fructose-galactose; and b, e, f, g, h, i) unknown peaks. Mobile phase: .0075N HzSO4; temperature, 65°C; flow rate, .7 ml/min; and injection volume, 10 μl.

did not occur in the standard solutions, which would indicate that uric acid was being transformed either by remaining enzymes in the extract, or in the presence of H2SO4, or both. The transformation did not occur during low temperature storage. Some of the samples were analyzed at a lower column temperature (50°C) to improve the separation of formic acid from the unknown peak, but no formic acid was detected. A shoulder of the peak corresponding to uric acid has been reported by Marsili et al. (8). This shoulder peak may be the peak that we observed, but not completely resolved. Other unknown peaks were detected. The first coeluting peaks (a in Figure 2) corresponded to phosphates and other unretained compounds. Between pyruvic and lactic acids, four peaks were observed (labeled c, d, e, and f in Figure 2); peaks c, d, and e were consumed at different rates during fermentation. Only peak d could be identified as fructose and galactose, and part of peak c could be glucose. Peak e disappeared completely during the first 5 h of fermentation, but peak f remained constant. Two other peaks appeared during fermentation. One peak appeared close to butyric acid (h in Figure 2), and the other had an approximate retention time of 27 min (peak i in Figure 2). Concentration of both increased during storage. During the first phase of fermentation, a relatively large peak was detected between lactic and uric acids, which disappeared afterward. We could identify none of the unknown peaks described, except for glucose, galactose, and fructose. Lactose was a very small peak, sometimes visible between orotic and citric acids. Succinic acid and urea were not detected in the milk or yogurt samples tested.

Figure 3 shows the evolution of pH and orotic, lactic, pyruvic, acetic, and propionic acids during yogurt fermentation and storage. Lactic acid was actively produced from the 2nd h of fermentation, and orotic and hippuric acids were consumed. Orotic acid is an intermediate product in the synthesis of nucleotides and a growth factor for yogurt starter cultures. Its decrease in concentration was around 30%. This decrease has also been reported in buttermilk (6) and yogurt (13). These and other researchers (2) reported a reduction of 45 to 48% in orotic acid content. Hippuric acid has been shown to be a precursor in the synthesis of benzoic acid by lactic acid bacteria in milk (9). Benzoic acid is a preservative in high acid foods. The disappearance of hippuric acid in yogurt may have implications for the shelf-life of yogurt. In our study, hippuric acid disap-

Figure 3. Evolution of A) pH (○) and orotic acid (○) and B) lactic (○), pyruvic (○), acetic (△), and propionic (△) acids during fermentation and storage of plain yogurt at 4°C.
peared during the first few hours of fermentation (from 20.1 µg/g in milk to 1.3 µg/g at 4 h). Pyruvic acid showed a maximum concentration around the 6th h of fermentation, decreasing afterward, possibly because pyruvic acid is an intermediate compound in different metabolic pathways and does not accumulate. During fermentation of buttermilk, the concentration of pyruvate increased continuously (6). In our study, pyruvate began to decrease after the 6th h of fermentation and continued during storage. Citric acid concentration (mean of 2.3 mg/g) did not change, but uric acid showed a slight decrease at the end of fermentation (from 34.7 µg/g in milk to 32.7 µg/g in yogurt). These observations are in accordance with those in other studies (12). Acetic and propionic acids increased during fermentation and storage.

ACKNOWLEDGMENTS

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REFERENCES