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ARTICLES

Development of a CE Method to Analyze Organic Acids in Dairy Products: Application to Study the Metabolism of Heat-Shocked Spores

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Organic acids are relevant in dairy products for nutritional reasons and because they contribute to the flavor and aroma. They are the major products of carbohydrate catabolism of lactic acid bacteria and nonstarter bacteria associated with milk. In several research and quality programs, it is very important to develop a rapid and sensitive method for their quantitative determination in dairy products to monitor bacterial growth and activity. A capillary electrophoresis method for the simultaneous determination of oxalic, citric, formic, succinic, orotic, uric, pyruvic, acetic, propionic, lactic, and butyric acid in less than 18 min has been developed. Various parameters affecting analysis, including capillary length, type, composition, and pH of the electrolyte have been optimized. Some alternatives are given to improve the separation of particular organic acids of special interest. Its application to analyze the quality of some dairy products has been investigated. In addition, the suitability of the technique to determine profiles of organic acids generated during the metabolism of heat-shocked spores has been demonstrated.

KEYWORDS: Organic acids; milk; capillary electrophoresis; heat-shocked spores

INTRODUCTION

Organic acids appear in dairy products as a result of the hydrolysis of milk fat (free fatty acids such as acetic or butyric), direct addition as acidulants (citric and lactic), normal bovine biochemical metabolism (citric, orotic, and uric), or bacterial growth (lactic, acetic, pyruvic, propionic, and formic). Also, they are the major products of carbohydrate catabolism of lactic acid bacteria (LAB). Their ability to produce acids with resulting pH reduction is the major factor in milk fermentations. The resulting acidity prevents the development of spoilage and pathogenic microorganisms, improving the hygienic quality of dairy products. However, it is important to note that the ability of LAB to inhibit undesirable bacteria depends not only on the reduction of pH but also on the sort of organic acids they produce (*1*).

The incidence of endospores in skim milk powder (SMP) is a problem because they survive pasteurization, evaporation, and spray-drying. Endospores are a risk in SMP because they are capable of germination and growth once the milk powder is rehydrated. Once vegetative, some spores are capable of hydrolyzing lipids, casein, and/or starch and fermenting lactose; all of these are detrimental to the quality of the SMP (*2*). Finding a rapid method for spore identification would be beneficial to screen the production industrially and determine SMP quality. Quantitative determination of organic acids is important to monitor bacterial growth and activity, for nutritional reasons, and because organic acids contribute to the flavor and aroma of dairy products (*3*).

Until a few years ago, organic acids have been commonly analyzed by chromatographic techniques. Most methods developed to analyze organic acids in dairy products are high-performance liquid chromatography (HPLC) methods (*1*, *3*, *4*). Capillary electrophoresis (CE) has emerged as a powerful separation technique that can provide high resolution and efficiency, offering great potential for rapid detection and quantification. Some CE methods have been developed to analyze organic acids in beer (*5*), tea infusions (*6*), citrus juice (*7*), and wines (*8*), but no CE methods to simultaneously separate the organic acids most frequently studied in dairy products (oxalic, citric, formic, succinic, orotic, uric, pyruvic, acetic, propionic, lactic, and butyric) have been found in the literature.

Because organic acids have little or no UV absorbance, detection must be accomplished by indirect UV by using a background electrolyte (BGE). Various BGEs have been

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reported (*9*); phthalate is one of the most popular. By applying a negative voltage, the organic anions migrate toward the detector situated at the anode end of the capillary. Addition of modifiers, such as hexadecyltrimethylammonium bromide (CTAB), reverse the direction of the electroosmotic flow (EOF) and promote the comigration of the analytes with the EOF, thus speeding up the analysis.

According to eq 1, the velocity (v_i) of compounds inside the capillary

$$v_i = (\mu_{ep} + \mu_{eo})E \quad (1)$$

and therefore the separation, depends on the electrophoretic mobility of the analyte (μ_{ep}) , the mobility due to the EOF (μ_{eo}), and the electric field (E) (*10*). These parameters are directly or indirectly affected by numerous variables, e.g., EOF is affected by pH, temperature, field strength, viscosity, current, and ionic strength, which in term may be affected by other variables, e.g., the current depends on capillary length and internal diameter, voltage applied, temperature, and ionic strength. Consequently, most of these parameters should be considered when optimizing a CE method.

The aim of this work was to develop a CE method for the simultaneous determination of 11 organic acids metabolically important in dairy products and most commonly cited in the literature, including oxalic, citric, formic, succinic, orotic, uric, pyruvic, acetic, propionic, lactic, and butyric acid. Various parameters affecting analysis, including capillary length, type, composition, and pH of the electrolyte, have been optimized. Its application to analyze the quality of some dairy products has been investigated. In addition, the suitability of the technique to determine the profile of organic acids generated during the metabolism of heat-shocked spores has been studied. This fact could be a very useful tool for early identification of spore former bacteria, once isolated from milk and dairy products.

MATERIALS AND METHODS

Instrumentation. All of the experiments were performed with a Bio-Focus 3000 Capillary Electrophoresis System (Bio-Rad Laboratories, Richmond, CA), consisting of a photodiode-array detector and two 32 position carousels (inlet and outlet). The system was controlled by a Gateway 2000 type P2/150 computer equipped with a Bio-Focus 3000 software for data collection and analysis. All fused-silica capillaries (75 μm i.d.) used in this work were purchased from Bio-Rad. All samples and buffers used were previously filtered through a 0.45 μm poly(vinylidene difluoride) membrane (GelmanSciences, Ann Arbor, MI) and degassed by centrifuging for 3 min at 14 000 rpm.

Reagents. All of the organic acids were obtained from Sigma (St. Louis, MO). Ultrapure water (18.2 MΩ) prepared by treating deionized water with a Barnstead/Thermolyne System (Dubuque, IA) was used to prepare all solutions. Individual stock solutions of each organic acid at 1000 ppm were prepared by dissolving the proper quantity in 4.5 mM H_2SO_4 containing 15 ppm of boric acid as internal standard (IS), except orotic and uric, which were prepared in 0.1 N NaOH. The reagents used to prepare the running buffers assayed were analytical or reagent grade: CTAB, potassium hydrogen phthalate (KHP), and sodium hydroxide were purchased from Sigma (St. Louis, MO); 2,6-pyridinedicarboxylic acid (PDC) and methanol were obtained from Aldrich (Milwaukee, WI).

Sample Preparation. *Dairy Samples.* Milk powder was manufactured with milk obtained from the Dairy Products Technology Center (DPTC, San Luis Obispo, CA) and pasteurized at 72 °C for 15 s. The concentration and drying processes of the milk were carried out as detailed by Caric (*11*). Briefly, the pasteurized milk was passed through the condenser (Marriott Walker Corporation, MI) to obtain concentrated milk with 45% of total solids. The concentrated milk was pumped into the spray dryer (Niro Inc., Columbia, MD) where the inlet and outlet air temperatures were 170 and 82 °C, respectively. The milk powder was reconstituted by dissolving the powder in water to 10% of the total solids concentration. Microbiological tests were carried on reconstituted samples. Control samples were incubated at 37 °C for 24 h, and test samples were inoculated from the DPTC collection to a final count between 2 and 3×10^6 spores/mL. Once the samples were prepared and incubated, the samples were immediately used for analysis. The rest of the dairy samples studied in this work (cheddar cheese and plain liquid yogurt) were purchased at local stores.

Organic Acids Extraction. According to González de Llano et al. (*1*), the extraction of organic acids from milk and cheese with 4.5 mM H_2SO_4 yields a satisfactory recovery, ranging from 96 to 103% for most of them. The extraction of the organic acids from the samples was made with 4.5 mM H_2SO_4, containing boric acid as IS. For liquid samples, 1 mL of sample was mixed with 5 mL of sample buffer for 30 min in the case of milk or for 1 min in the case of whey permeate and culture media. After that, the samples were centrifuged and the supernatant was filtered and degassed as explained above. For solid or semisolid samples, the mixture was stirred for 45 min.

Heat-Shocked Spores Inoculation and Sampling. To analyze the suitability of the CE method to study the metabolism of bacteria, approximately the same quantity of heat-shocked (80 °C for 12 min) spores of eight strains [1, *Bacillus licheniformis* 14580; 2, *Bacillus subtillis* 23059; 3, *Bacillus cereus*; plus five strains of *Bacillus* from the DPTC (Cal Poly University, San Luis Obispo, California) collection, 4, SL 3; 5, CL 6; 6, CL 10; 7, CH 3; and 8, CL1] were inoculated separately in bottles containing aerobic media consisting of a supplemented tryptic soy broth and additional complex amino acids and carbohydrates substrates (BacT/Alert, Organon Teknika Corp., Durham, NC). The bottles were incubated at 40 °C in a BacT/Alert Microbial Detection System connected to a computer equipped with a BacT/Alert Data Management System for data collection. BacT/Alert is a totally closed automated system for incubating, shaking, and monitoring culture bottles for microbial growth. The bottom of the culture bottles has a colored CO_2 sensor. As microorganisms grow and produce CO_2, the sensor changes its color and an external monitor detects the change from dark green to yellow. The readings are transmitted to a computer and a graph (reflectance units vs time) is created for each bottle. A mark appears on the curve at the time the bottle has been considered positive.

For the analysis of organic acids for each strain, samples from BacT/Alert were taken at three different times corresponding to the points of the curve where the production of CO_2 starts to increase, the bottle is considered positive, and finally the point of the curve where the slope changes again because no more increase of CO_2 production is detected. The extraction and analysis of organic acids were carried out as explained above. A mixture of standard organic acids at 50 ppm was added to some samples for the identification of the peaks. Quantification was made with standards of each acid; boric acid was used as IS. The calibration curves were calculated taking into account the purity of the standards; the area of each peak/retention time was used as a response. All of the samples were analyzed in duplicate.

Electrophoretic Procedures and Conditions. Unless specified otherwise, the final conditions used to analyze the samples are as follows. The BGE was prepared daily with 4.4 mM KHP and 0.27 mM CTAB. The pH of the buffer was adjusted at 11.2 with 1 M NaOH. The separations were carried out on fused-silica capillaries with 105 cm of effective length × 75 μm i.d. Prior to first use, a new capillary was pretreated with 0.1 N NaOH for 10 min, followed by water for 10 min and BGE for 10 min. Before each run, the capillary was preconditioned with run electrolyte for 3 min. For short periods of time, the capillary was stored full with BGE with both ends submerged in electrolyte reservoirs to avoid salt precipitation; for longer periods, the capillary was conditioned for 3 min with run electrolyte and dried by passing nitrogen for 1 min at 100 psi before storing it.

The sample was injected by hydrodynamic injection at pressure (5 psi) × time (s) = 2. The separation was performed at −20 kV, and the capillary was thermostated at 30 °C. The wavelength for indirect UV detection was selected at 200 nm, and the signal with negative peaks was flipped to obtain a more familiar electropherogram to integrate and process.

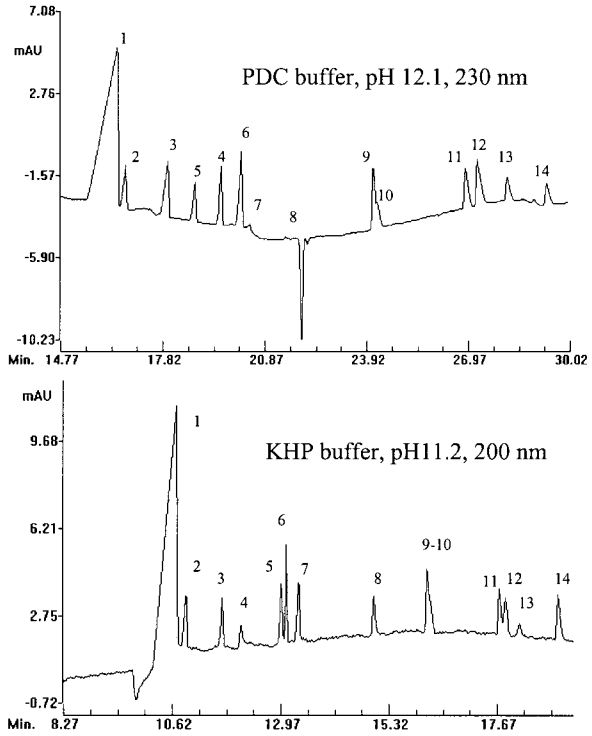

Figure 1. Effect of the type of BGE used for the running buffer. Test mixture [hydrodynamic injection at pressure (5 psi) × time (s) = 10 of the test mixture at 50 ppm in the case of PDC buffer; pressure (5 psi) × time (s) = 2 of the test mixture at 20 ppm in the case of KHP] in 4.5 mM H_2SO_4 containing boric acid as IS: (1) sulfuric; (2) oxalic; (3) unknown; (4) citric; (5) formic; (6) succinic; (7) orotic; (8) uric; (9–10) acetic–pyruvic; (11) propionic; (12) lactic; (13) IS; and (14) butyric.

RESULTS AND DISCUSSION

Separation Optimization. Several parameters that affect the separation have been studied.

Running Buffer Selection. The choice of the BGE is the most important in developing a CE method employing indirect UV detection. In the indirect absorbance method, an absorbing BGE, which provides a background UV absorbance, comigrates with the mixture of analytes, which do not absorb at the wavelength set for the detector. The BGE with a mobility matching those of most of the analytes would give a better separation and resolution (*9*). Two BGEs including KHP and PDC were evaluated with the test mixture consisting of the organic acids indicated above. Twenty millimolar PDC buffer was prepared by adding 0.5 mM CTAB and adjusting the pH at 12.1 as recommended by Soga and Ross (*12*). A good resolution was obtained for the latest peaks of the electropherogram, including acetic and pyruvic acids. Because orotic and uric acids absorb at the wavelength selected for this buffer, the sensitivity was drastically decreased for orotic acid, and uric acid appeared as a negative peak. However, if the CE system lets one select two different wavelengths (λ = 230 and 300 nm), these two compounds can be easily detected at 300 nm, obtaining for the same analysis one electropherogram at 300 nm for them and another at 230 nm with the rest. In this work, because of the slightly high concentration used for the BGE (20 mM), large quantities of 1 N NaOH were necessary to adjust the pH to 12.1. Consequently, at the voltage reported (30 kV) to run the samples, the current was excessively high (>100 μA), which increased the noise of the baseline. To solve that problem, the voltage was assayed at 28 and 25 kV, but the intensity remained high, and the resolution was poorer. At 20 kV (**Figure 1** shows the electropherogram obtained for the test mixture using the PDC buffer at 20 kV), the analysis time was long (30 min) and the baseline notably wavy. The resolution of the compounds was better at high voltages; therefore, in a trail to use high voltage to separate the analytes, the concentration of PDC was decreased to 10 mM to avoid extreme currents. Although the current became more acceptable (60 μA at 28 kV), a poorer resolution in both citric−formic and propionic−lactic pairs was detected and acetic−pyruvic acids coeluted. In the same way, concentration of CTAB was assayed at 0.5 and 0.25 mM, but the results practically did not change. However, this buffer (20 mM PDC and 0.5 mM CTAB at pH 12.1) was tested in another CE system that controlled with great accuracy the run temperature and used a fluoroorganic fluid as the coolant, obtaining good results for all of them and good baseline. The coolant system is very important to allow a quick heat dissipation on the capillary, to keep the current constant, and therefore to obtain a good baseline.

Because phthalate acid is one of the most popular BGE to analyze organic acids (*8, 13*) and because it gave the best overall separation of the test mixture in our CE system, KHP was chosen for further investigation. Most of the phthalate work reported utilized 254 nm as the detection wavelength. However, our CE system is equipped with a diode array detector, which allowed us to set it at 200 nm. At this wavelength, the absortivity was higher and the sensitivity of the method was improved more than two times.

pH of the Running Buffer. The electrophoretic mobility of acids is strongly dependent on the pH of the running buffer. The pH of the KHP buffer was varied from 5.6 to 12.2 with 0.2 increments. To study the effect of pH, a mixture of oxalic, citric, formic, succinic, acetic, propionic, lactic, and butyric acids was tested. At pH 5.6, the oxalic acid was not detected (see **Figure 2**). This peak migrates close to the peak of sulfate, which should be added at 4.5 mM to the test mixture because 4.5 mM sulfuric acid was used to extract the organic acids from the samples and it would appear in the electropherograms. Also, at this pH, formic acid comigrated with citric acid while the resolution was complete for the rest. At pH values closer to 7.0, oxalic acid started to appear but it suffered from severe broadening. Also, there was no resolution between formic and citric acids, and propionic and lactic acids were poorly separated. At pH 11.0, all compounds tested were resolved satisfactorily. In the electropherogram of **Figure 2** also appear the rest of the organic acids of interest (orotic, uric, and pyruvic), which were not in the test mixture initially but were added later. However, an unknown peak (peak 3) appeared in the electropherogram close to citric acid, making quantification of this acid more difficult. This peak only appeared in the electropherogram when high pH value running buffers were tested, due to the high quantity of NaOH added to adjust the pH of the electrolyte. In fact, it did not appeared in the electropherograms corresponding to pH values 5.6 and 6.8. To improve the separation between citric and the unknown peak, the pH of the buffer was changed to 11.2. In this form, a very good separation of these two peaks was achieved (see **Figure 1**). This fact is especially important in the analysis of milk samples, where normal concentration of citric acid has been reported around 0.9−2.2 mg/mL depending on the literature (*1*, *3*, *4*). Good resolution was obtained for the rest of the organic acids (orotic, uric, and pyruvic) added to the test mixture, even though propionic and lactic acids were not separated completely when their concentration values were 20 ppm or higher and pyruvic and acetic acids comigrated at concentration values higher than 10 ppm.

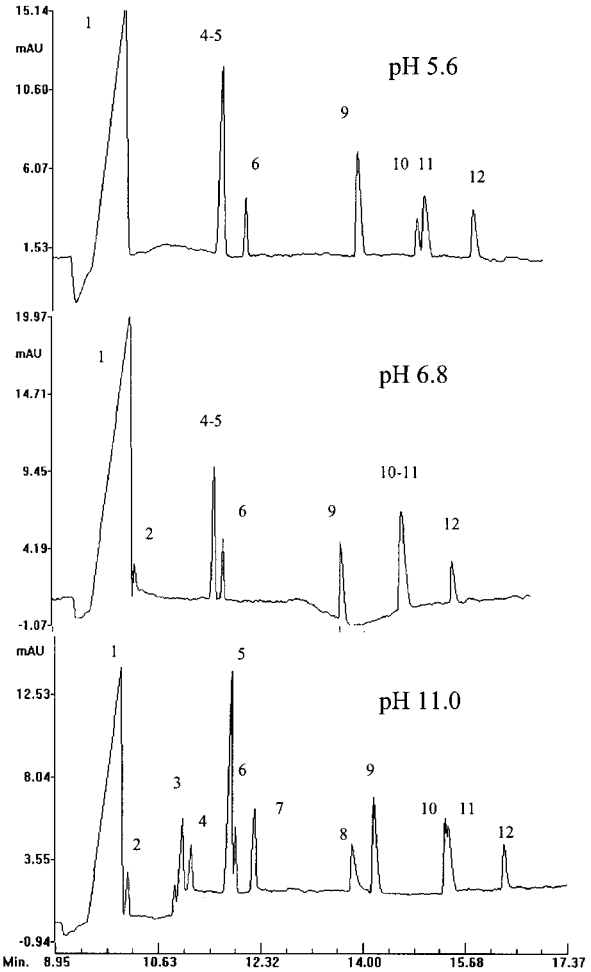

Figure 2. Effect of the pH. (1) Sulfuric; (2) oxalic; (3) unknown; (4) citric; (5) formic; (6) succinic; (7) orotic; (8) uric; (9) acetic–pyruvic; (10) propionic; (11) lactic; and (12) butyric.

Concentration of BGE and EOF Modifier (EOFM). The concentration of the electrolyte can have a big impact on the baseline noise, sensitivity, and linear dynamic range (*10*). Concentration of phthalate between 1 and 25 mM has been reported in the literature. To find the optimal KHP concentration, that range was analyzed. The highest levels were discarded because high ionic strength in buffer caused high Joule heating, and the baseline became noisy. The optimum concentration was found at 4.4 mM.

CTAB was selected because it has been frequently used as EOFM to reverse the direction of the EOF, providing the best resolution between the test analytes (*14*). The effect of the modifier should be proportional to its concentration up to when an optimal concentration is reached. At the highest concentration of CTAB tested (5 mM), propionic and lactic acid comigrated, and practically the rest of the compounds were not affected. The concentration of CTAB was decreased up to when the optimal was determined at 0.27 mM.

Capillary Length. A decrease in efficiency when switching from 50 to 75 μm i.d. capillaries has been reported (*14*). However, a 75 μm i.d. capillary provides about 1.5-fold increase in sensitivity due to an increased detection path length. Because sensitivity was an issue for our applications, a 75 μm i.d. capillary was chosen. Also, a 105 cm total length capillary was used to optimize resolution. After optimization of the technique, shorter capillaries (50 and 90 cm) were tested in order to maximize the speed of the analysis. As shown in **Figure 3** (electropherogram a), when the time of the analysis is shortened, it results in poor resolution as can be observed, making it very difficult to quantify, even when 10 kV was used as the run voltage (**Figure 3**, electropherogram b). That was especially important in milk samples where the highest peaks are citrate and phosphate (see **Figure 3**, electropherogram d), and with these capillaries lengths, both compounds were not completely resolved. Nevertheless, the fact that the highest peaks in milk are citrate and phosphate could be an advantage. By extracting 1 mL of milk with 20 mL of sample buffer, the quantity of other organic acids present in the milk would be lower than the

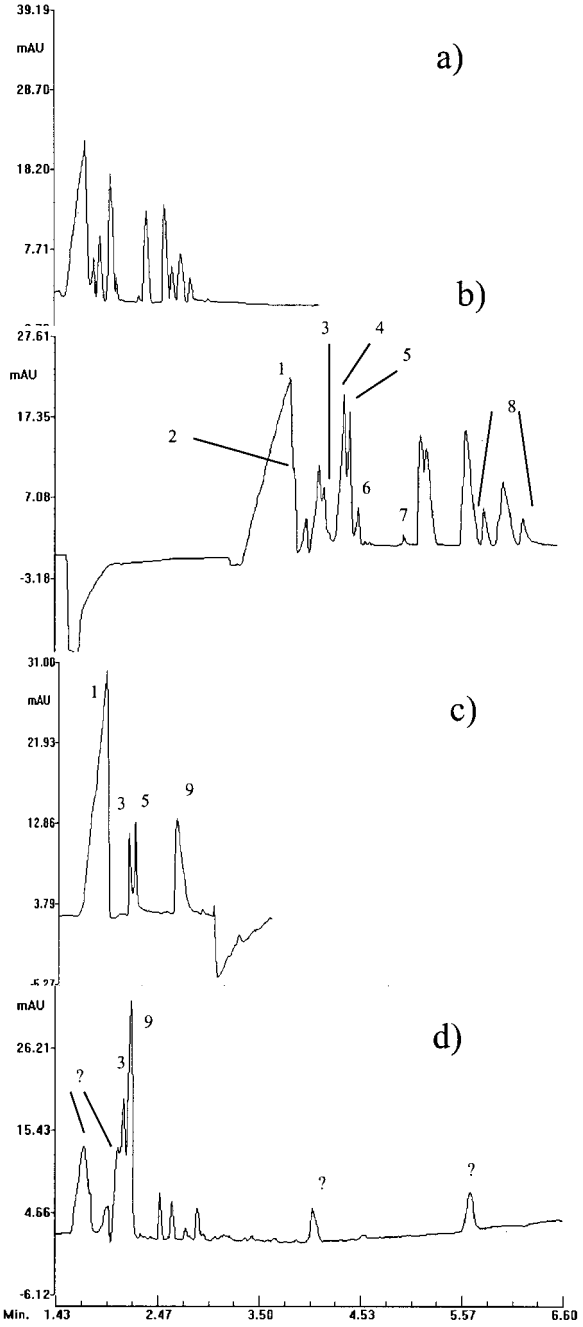

Figure 3. Effect of capillary length (50 cm total length). (**a**) Fifty parts per million standard mixture; (**b**) 50 ppm standard mixture analyzed at −10 kV; (**c**) milk extracted with 4.5 mM H_2SO_4, containing succinic acid as IS and analyzed at pH 6.2 and −20 kV; (**d**) whey permeate in water (5:1). (1) Sulfuric; (2) oxalic; (3) citric; (4) formic; (5) succinic; (6) orotic; (7) uric; (8) peaks appeared when sample buffer was run; and (9) phosphate.

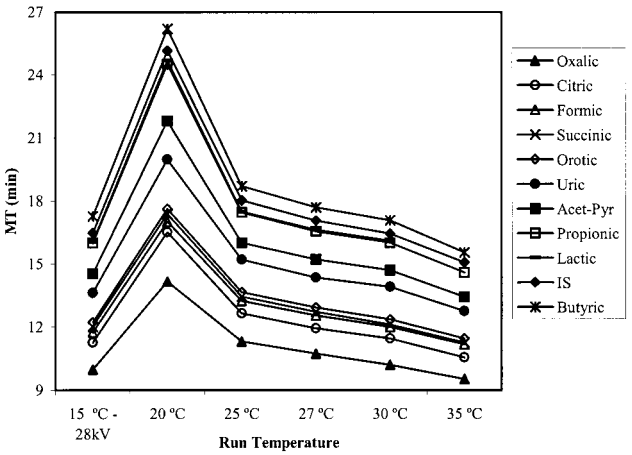

Figure 4. Effect of the run temperature on migration times. Run voltage, –20 kV, except sample run at 15 °C.

detection limit of the CE method. Only two peaks, corresponding to citric and phosphate, would appear in the electropherogram. In this form, we could use a 50 cm capillary to analyze the quantity of these compounds in less than 3 min (**Figure 3**, electropherogram c). The pH of the running buffer had to be adjusted at 6.2 to avoid coelution, and we could add succinic acid to the sample as IS. The knowledge of the quantity of these compounds is very interesting because their distribution between soluble and colloidal phases of milk and their interactions with milk proteins are important factors in the stability of dairy products (*15*).

Effect of the Run Temperature. Because both the electrophoretic migration velocity and the EOF velocity are affected by the column temperature, the control of ambient conditions can have a significant influence on migration time variability. Increasing capillary temperature can shorten separations much the same as increasing the applied voltage (*16*). In most of the works published, the capillary was thermostated to 15−20 °C. We tested various temperatures (**Figure 4**); at 20 °C, the analysis time was long and the butyric acid passed the zone of the detector close to minute 27. We evaluated higher temperatures to shorten the run time. At 30 °C, we were able to shorten the analysis to 17 min, obtaining practically the same resolution as decreasing the temperature and without varying the relative migration time of the peaks. At 35 °C, the separation of formic and succinic was worse and no resolution between propionic and lactic was achieved. Also, we ran the test mixture at 15 °C, but we increased the run voltage up to 28 kV to counteract the effect of the lower temperature. Nevertheless, the migration times were similar to those obtained at 30 °C and the baseline became wavier. Therefore, 30 °C was chosen as the optimum temperature for our analysis.

Precision and Linearity. Because the recovery in the extraction of organic acids from milk and cheese with 4.5 mM H_2SO_4 has been reported in the literature (*1*), our proposal was not to validate the method of extraction. However, we shortened the extraction time, as indicated in the Materials and Methods section, because we did not find any difference in the results when using those longer times recommended by the mentioned authors.

The reproducibility and linearity of the analytical technique was determined (**Table 1**). The precision (expressed in terms of relative standard deviation (RSD)) for five determinations at 40 ppm was better than 0.7% for relative migration times and better than 7% for peak area to migration time ratios, except for uric and citric acids (RSD ∼ 10%). These showed the smallest peak areas and therefore were more difficult to integrate. It has been suggested (*17*) that the uric acid could be transformed in the presence of H_2SO_4 when maintained for more than 1 h at 21 °C, which could explain the higher RSD values found for this compound. In fact, better RSD values were obtained when we kept the sample frozen until its analysis.

Table 1. Regression Equations for the Calibration Curves and Results of the Analysis of the Linearity and Precision

compd	regression equation	R^2 [a]	RSD[b] (MT)	RSD[b] (area)
oxalic acid	$y = 0.0783x - 0.0955$	0.9945	0.6	7.6
citric acid	$y = 0.0888x - 0.2561$	0.9923	0.4	10.8
formic acid	$y = 0.1368x - 0.2478$	0.9952	0.3	5.4
succinic acid	$y = 0.0973x - 0.1294$	0.9981	0.3	6.4
orotic acid	$y = 0.0235x - 0.0332$	0.9956	0.3	6.4
uric acid	$y = 0.0108x + 0.0717$	0.9861	0.7	10.8
prop–acetic acids	$y = 0.0854x - 0.2454$	0.9948	0.2	5.1
propionic acid	$y = 0.0582x + 0.0203$	0.9923	0.1	5.6
lactic acid	$y = 0.0894x - 0.3636$	0.9908	0.1	3.7
butyric acid	$y = 0.0839x - 0.1282$	0.9978	0.1	5.6

[a] R^2 = coefficient of determination. [b] RSD values for migration times (MT) and for relative peak areas (area) in percent.

The results obtained in this work were equivalent to those reported for Wu et al. (*9*) or Galli et al. (*18*) using phosphoric acid as BGE to analyze some of these organic acids and for Chen et al. (*14*) who obtained a RSD of 6% at 50 ppm using phthalate as electrolyte.

The calibration curves were created for each acid within the range of 5−100 ppm, and boric acid was used as IS. The data points from calibration curves were subjected to least-squares regression analysis and the slope (*a*), intercept (*b*), and coefficient of determination (r^2) for the analytes were calculated. The linearity of the present method was good with r^2 values better than 0.99, except for uric acid ($r^2 = 0.986$).

Applications of the Method. The proposed method was applied to analyze some dairy samples but also to analyze the growth of heat-shocked spores.

Dairy Products. The optimized method was applied to the determination of some commercial samples (**Figure 5**). Milk powder was reconstituted, and its organic acids were extracted and analyzed as explained. As expected, one of the biggest peaks was that corresponding to citric acid; levels of citrate in milk between 0.9 and 2.2 g/L have been reported in the literature (*1*, *3*, *4*). A big peak appeared after citrate at the same retention time as the formic acid did. However, the concentration of formic acid in the milk is lower than 40 mg/L (*1*, *3*); therefore, that peak should be another compound. Other compounds were investigated, and phosphoric acid appeared closer to formic. Because of the fact that the normal concentration of phosphate in milk is around 2 g/L and its migration time is similar to the retention time of the formate, probably the big peak of phosphate caused the comigration of both phosphate and formate.

Also, **Figure 5** shows the electropherograms of powder milk reconstituted and incubated at 37 °C for 24 h (panel **b**) and powder milk reconstituted but also inoculated with spores (panel **c**). As expected, a growth of organic acids such as succinic, and especially acetic−pyruvic and lactic, was observed. The metabolism of the carbohydrates present in the milk by the bacteria generates organic acids, which are detectable by this technique. Some of them (pyruvic, acetic, and lactic) have been considered as an index of bacterial growth (*3*). Also, other unidentified peaks that appeared in the electropherogram could be amino acids released from the proteolysis of caseins by

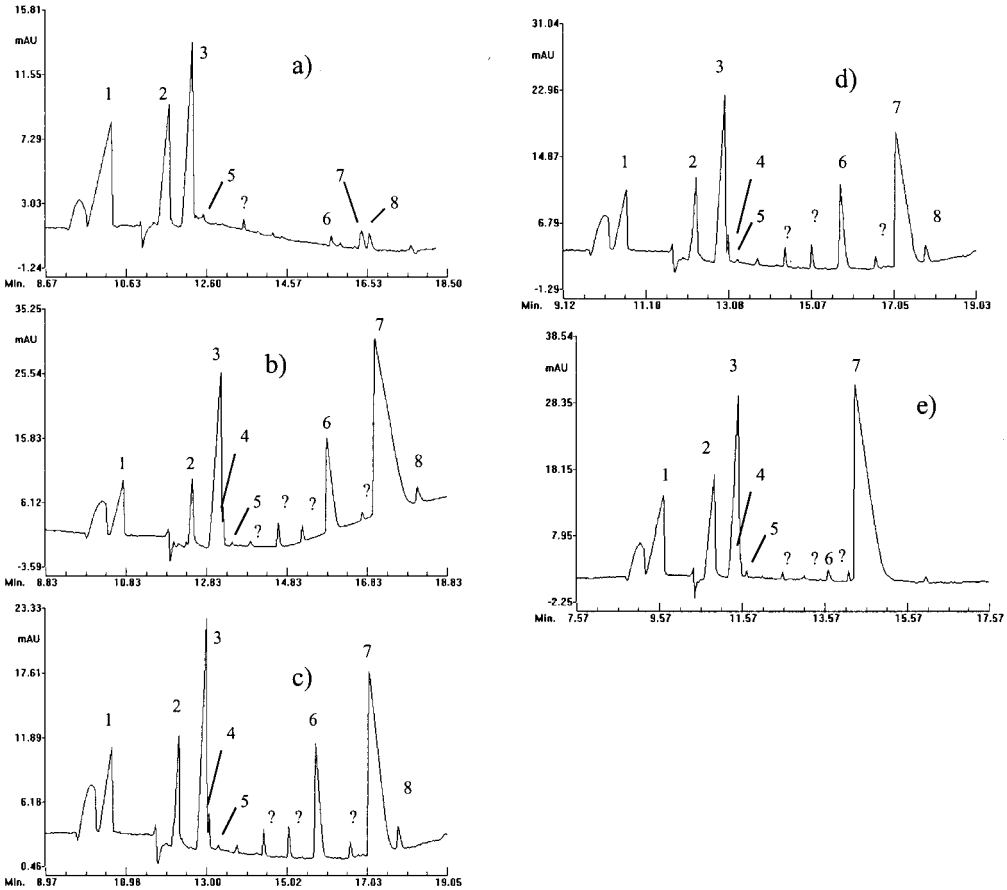

Figure 5. Electropherograms of (**a**) milk powder reconstituted, (**b**) milk powder reconstituted and incubated for 24 h at 37 °C, (**c**) milk powder reconstituted and inoculated with spores and incubated for 24 h at 37 °C, (**d**) 3 month old Cheddar cheese, and (**d**) liquid yogurt. (1) Sulfuric; (2) citric; (3) phosphate + formic; (4) succinic; (5) orotic; (6) acetic–pyruvic; (7) lactic; and (8) IS.

bacterial activity. Amino acids such as Asp, Glu, Tyr, or Gly have been detected between succinic and lactic acid when PDC at pH 12.1 is used as BGE (*12*). As shown in **Figure 5b**,**c**, both electropherograms had a similar profile. Despite not having counted the bacteria present in both samples, we can speculate that in case the spores germinated during the process (manufacturing process, reconstitution, and incubation), either their presence was not high enough to make differences with the control or their metabolism was similar to those bacteria present in the control.

Two other electropherograms corresponding to the organic acids extract of 3 month old cheddar cheese (**Figure 5c**) and liquid yogurt (**Figure 5d**) have been included. Taking into account the importance of organic acids for the flavor, this method could be very useful to characterize the profile and quantity of these organic acids and to correlate it with the characteristic taste found in this kind of product, especially different grades and types of sour taste.

Growth of Heat-Shocked Spores. Bacterial spores exhibit no detectable metabolism and can withstand extremes of pH, temperature, desiccation, humidity, and radiation, which would rapidly kill the vegetative cell from which they arise (*19*). Because spores can germinate and therefore hydrolyze lipids, caseins, and/or ferment lactose, finding a method for spore identification, and at the same time allowing early determination of their metabolism, would be very beneficial for quality control in the dairy industry.

As explained in the Materials and Methods, eight strains were incubated in BacT/Alert system for care of handling and monitoring of growth, and samples of each strain were taken at three different times corresponding to (i) the points of the curve where the production of CO_2 starts to increase, (ii) the bottle is considered positive, and (iii) no more increase of CO_2 is detected. **Table 2** shows the sampling times for each strain. An example of the electropherograms obtained for the strainnumber 7 at the three sampling times can be observed in **Figure 6**.

Table 2. Sampling Times for the Blank (Sterile BacT/Alert Culture Media) and Eight Heat-Shocked Spores Incubated at 40 °C

		sampling time (h)		
	no.	time 1	time 2	time 3
blank	0	7	10	13
B. licheniformis 14580	1	7	10.5	13
B. subtillis 23059	2	7	9.5	12
B. cereus	3	7	11	14.5
B. SL 3[a]	4	8	11	16
B.CL 6[a]	5	7	10	13
B. CL 10[a]	6	7	9.5	13
B. CH 3[a]	7	8	11	16
B. CL1[a]	8	8	11.5	16

[a] Strains of Bacillus from the DPTC (Cal Poly University, San Luis Obispo, California) collection.

A blank sample containing only sterile media was incubated as well as the rest and sampled after 7, 10, and 13 h of incubation. For each sampling date (times 1, 2, and 3, see **Table 3**), the concentration of each organic acid of the eight strains was calculated by subtracting the concentration of the organic acid present in the corresponding blank sample. All of the organic acids except orotic acid were detected and quantified

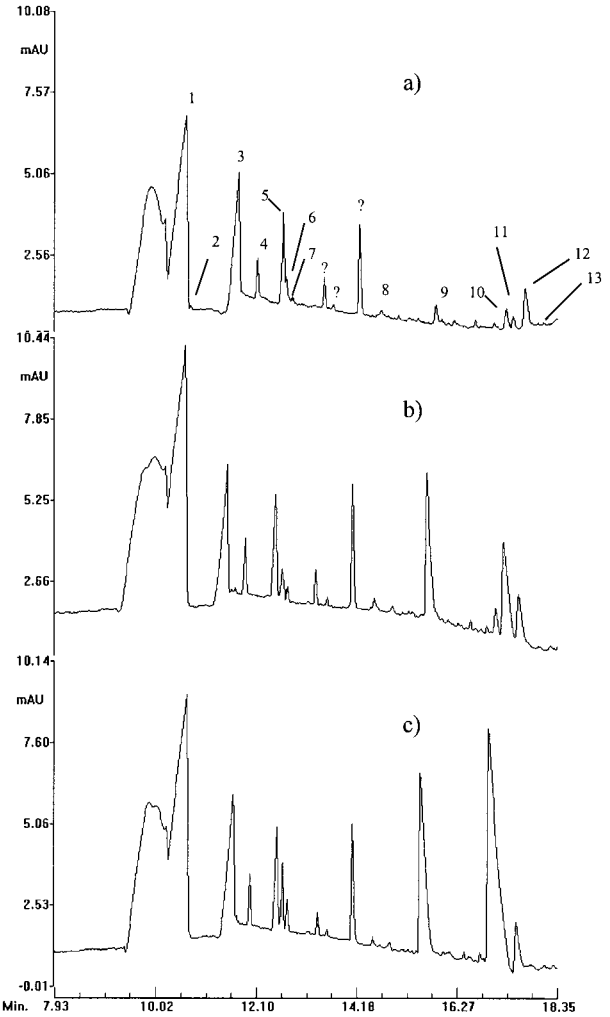

Figure 6. Electropherograms of BacT/Alert aerobic media inoculated with spores of strain number 7 (*Bacillus* CH 3) and incubated in BacT/Alert system. Electropherograms after (**a**) 8, (**b**) 11, and (**c**) 21 h of incubation. (1) Sulfuric; (2) oxalic; (3) unknown; (4) citric; (5) phosphate; (6) formic; (7) succinic; (8) uric; (9) acetic–pyruvic; (10) propionic; (11) lactic; (12) IS; and (13) butyric.

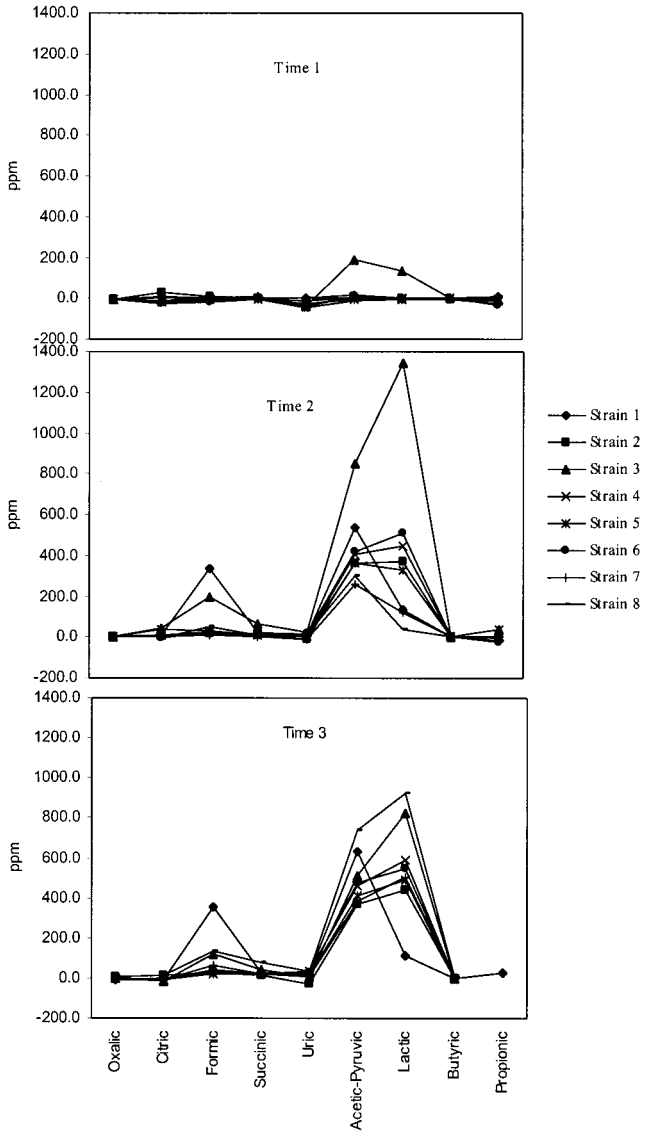

Figure 7. Quantification of organic acids of eight strains of heat-shocked spores incubated at 40 °C in BacT/Alert media for different periods of time.

in the BacT/Alert media. At time 1, the concentrations of oxalic, citric, formic, succinic, uric, acetic−pyruvic, lactic, and butyric present in the blank sample were 11.0 ± 1.6, 69.1 ± 5.2, 47.9 ± 1.4, 27.2 ± 0.4, 67.7 ± 9.2, 66.3 ± 0.5, 49.2 ± 9.2, 38.2 ± 0.3, and 21.2 ± 0.5 ppm, respectively. Because no culture was inoculated in the blank, their concentrations practically remained the same during the period of time studied. In the case of bacterial cultures, not a notable variation of the concentration of organic acids was observed (**Figure 7**). It seems to indicate that no appreciable metabolism and, therefore, detectable growth by the BacT/Alert system has happened during this period of

Table 3. Concentration (ppm) of Organic Acids from Control (Blank) and from Eight Heat-Shocked Spores Incubated at 40 °C in BacT/Alert Culture Media until the System Considered the Bottle Positive (Sampling Time 2)

strain no.	oxalic	citric	formic	succinic	uric	acet–pyru	propionic	lactic	butyric
blank 1[a]	11.0 ± 1.6	69.1 ± 5.2	47.9 ± 1.4	27.2 ± 0.4	67.7 ± 9.2	66.3 ± 0.5	49.2 ± 9.5	38.2 ± 0.3	21.2 ± 0.5
blank 2[a]	5.9 ± 0.1	84.2 ± 15.7	53.7 ± 0.8	26.4 ± 0.1	78.0 ± 13.5	74.0 ± 3.1	41.1 ± 0.1	40.7 ± 0.3	21.8 ± 0.1
blank 3[a]	6.6 ± 0.7	71.6 ±	41.0 ± 0.1	25.0 ± 0.1	60.2 ± 9.5	60.9 ± 0.9	41.2 ± 0.1	44.5 ± 6.9	20.9 ± 0.7
1[b]	6.5 ± 0.2	90.1 ± 9.1	389.4 ± 10.7	46.2 ± 0.6	89.5 ± 20.3	609.8 ± 9.3	84.2 ± 3.6	171.9 ± 0.8	21.8 ± 0.4
2[b]	5.6 ± 0.3	86.0 ± 8.6	72.6 ± 0.3	36.2 ± 0.4	64.8 ± 1.8	435.3 ± 8.5	78.3 ± 6.5	412.6 ± 16.1	20.5 ± 0.6
3[b]	7.1 ± 1.2	127.4 ± 13.7	252.5 ± 0.7	92.8 ± 1.6	98.1 ± 10.5	921.8 ± 30.7	120.6 ± 12.9	1382.6 ± 15.1	23.6 ± 1.0
4[b]	4.8 ± 0.1	123.3 ± 19.5	82.0 ± 1.7	37.8 ± 0.3	82.9 ± 1.4	482.5 ± 6.7	114.6 ± 4.6	484.4 ± 9.4	20.5 ± 0.5
5[b]	9.3 ± 0.3	119.5 ± 16.9	73.9 ± 0.7	46.4 ± 0.1	87.4 ± 2.2	563.9 ± 20.9	113.0 ± 4.3	686.7 ± 42.1	19.5 ± 0.4
6[b]	nd[c]	81.7 ± 9.5	73.7 ± 0.4	43.6 ± 0.1	91.6 ± 6.0	495.6 ± 8.6	51.7 ± 4.4	553.7 ± 2.8	21.5 ± 0.7
7[b]	nd[c]	86.0 ± 6.3	63.2 ± 1.7	31.3 ± 0.8	66.5 ± 3.2	330.4 ± 7.8	65.3 ± 1.5	158.6 ± 6.1	20.4 ± 0.3
8[b]	nd[c]	76.1 ± 8.4	104.4 ± 1.5	34.6 ± 0.3	83.3 ± 17.8	372.2 ± 3.3	59.8 ± 5.8	80.6 ± 0.1	20.4 ± 0.4

[a] Blank at sampling times 1, 2, and 3. [b] Strains of *Bacillus* from the DPTC collection (see **Table 2**). [c] Not detected.

time, except in the case of strain 7, where concentrations of acetic−pyruvic and lactic acids were clearly higher than the rest. Acetic acid may be produced by the fermentation of lactose, lactic, and citric acids, while pyruvic and lactic acid are formed by the fermentation of lactose but also of citric acid (*20*). It seems to be that the first source used by this strain has been lactose, since not a large decreasing of the citric acid was detected and levels of acids formed were high.

When the BacT/Alert system considered the bottles positive (sampling time 2, see **Table 3**), significant changes in acid concentration were appreciated, especially for formic acid (in strains 1 and 3) and acetic−pyruvic and lactic acids. The production of acetic−pyruvic and lactic acids by strain 3 increased highly, with values (obtained after subtracting the concentration present in the blank) of 847.8 ± 30.7 and 1342.8 ± 15.1 ppm, respectively. Strain 1 showed a profile characteristic and different than the rest of heat-shocked spores. Increase of the concentration of formic and acetic−pyruvic acids was observed clearly for these bacteria, while curiously concentration of lactic acid was clearly lower than the rest and remaining the same until sampling time 3. This could be due to a different metabolism by this strain or because the lactic acid generated was rapidly metabolized. The concentration of lactic acid of strains 7 and 8 at time 2 seems to be lower than the rest (117.9 ± 6.1 and 39.9 ± 2.1 ppm, respectively, after subtracting the concentration of the blank), but 5 and 4.5 h later, the production increased drastically, especially in the case of strain 8 with 926.4 ± 50.5 ppm of lactic acid at time 3. The production of organic acids such as lactic acid by these two bacteria seems to be lower than the rest, perhaps due to a slower metabolism. In fact, the time passed since the BacT/Alert system detected the change of the sensor, until no more production of CO_2 is detected, was longer for these strains (**Table 2**). The rest of the bacteria (2, 4, 5, and 6) had similar profiles at times 2 and 3, with slightly higher or lower levels. We could speculate that these four strains had similar metabolisms as related to production and dissimilation of organic acids.

These data must be interpreted with precaution. Bacteria have different metabolisms, and the BacT/Alert detects the production of CO_2 released from their metabolisms. Probably, when different strains are inoculated separately in bottles containing BacT/Alert media and the respective bottles are considered positive by the system, the number of bacteria of each strain will not be necessarily the same due to different metabolic rates of CO_2 production. Therefore, positive bottles with different strains have a different number of bacteria. Consequently, to correlate the quantity of CO_2 (or organic acids) produced/bacteria unit, the recounts of bacteria should be done along the period of incubation and the BacT/Alert system should be calibrated. Analyzing the bottles (containing a standardized media) when they are considered positive (since at the point where the bottle is considered positive for a particular strain is supposed to be present equivalent quantity of bacteria, the profile and quantity of organic acid generated should be similar), the CE procedure offers an alternative and complementary method to evaluate and identify isolated spore former bacteria.

In summary, organic acids analysis by CE appears to be a single alternative to other analytical techniques, being viable for many dairy matrixes. CE provides the additional advantages of low solvent consumption (milliliters per day vs liters per day for HPLC), no hazardous solvents, and low costs as compared with others (more than 1000 analyses were run with the same capillary without loss of resolution; HPLC columns must be deeply cleaned and regenerated after a few analyses). CE is a useful technique for the simultaneous separation and quantification of organic acids in dairy products such as milk, cheese, powder milk, buttermilk, and yogurt. The procedure offers simple sample preparation for the analysis of the organic acids most frequently found in dairy products and metabolically important (oxalic, citric, formic, succinic, orotic, uric, acetic, pyruvic, propionic, lactic, and butyric acids), even though acetic and pyruvic acid were not separated completely. Nevertheless, some different alternatives are given (using PDC as BGE or decreasing the pH of the running buffer) to separate these compounds, in case they are of special interest.

The proposed technique has been used to study and quantify the evolution of organic acids present in a media inoculated with different heat-shocked spores and incubated for different periods of time. The CE method allowed us to establish differences in the profiles of organic acids for each strain providing valuable information, which could be used not only to study their metabolism but also to obtain "reference fingerprints", very useful for the identification of bacteria. In the same form, this technique could be very valuable to study the metabolism and degradation products such as organic acids by bacteria (e.g., starter cultures) inoculated in different media. To use the proposed method for the applications indicated above, a quantitative basis per some unit of bacteria growth should be done. In fact, our target for forthcoming work is to correlate the production or degradation of organic acids per unit of bacteria.

LITERATURE CITED

(1) González de Llano, D.; Rodríguez, A.; Cuesta, P. Effect of lactic starter cultures on the organic acid composition of milk and cheese during ripening-analysis by HPLC. *J. Appl. Biotechnol.* **1996**, *80*, 570−576.

(2) Bellenson, E. Y.; Frazier, C. L.; Kitts, C. L.; Jimenez-Flores, R. The influence of Preheat Treatments on mesophilic endospores counts from California milk powder. *J. Food Sci.* **1998**, *80* (Suppl. 1), 137.

(3) Marsili, R. T.; Ostapenko, H.; Simmons, R. E.; Green, D. E. High performance liquid chromatographic determination of organic acids in dairy products. *J. Food Sci.* **1981**, *46*, 52−57.

(4) Mullin, W. J.; Emmons, D. B. Determination of organic acids and sugars in cheese, milk and whey by high performance liquid chromatography. *Food Res. Int.* **1997**, *30*, 147−151.

(5) DeVries, K. J. Determination of organic acids in beer by capillary electrophoresis. *J. Am. Soc. Brew. Chem.* **1993**, *51*, 155−157.

(6) Horie, H.; Yamauchi, Y.; Kohata, K. Analysis of organic anions in tea infusions using capillary electrophoresis. *J. Chromatogr. A* **1998**, *817*, 139−144.

(7) Saavedra, L.; García, A.; Barbas, C. Development and validation of a capillary electrophoresis method for direct measurement of isocitric, citric, tartaric and malic acids as adulteration markers in orange juice. *J. Chromatogr. A* **2000**, *881*, 395−401.

(8) Levi, V.; Wehr, T.; Talmadge, K.; Zhu, M. Analysis of organic acids in wines by capillary electrophoresis and HPLC. *Am. Lab.* **1993**, *January*, 29−32.

(9) Wu, C. H.; Lo, Y. S.; Lee, Y.-H.; Lin, T.-I. Capillary electrophoresis determination of organic acids with indirect detection. *J. Chromatogr. A* **1995**, *716*, 291−301.

(10) Oda, R. P.; Landers, J. P. Introduction to Capillary Electrophoresis. In *Handbook of Capillary Electrophoresis*; Landers, J. P., Ed.; CPC Press: London, UK, 1994; pp 10−42.

(11) Carić, M. Milk Powder: General Production. In *Concentrated and Dried Dairy Products*; Hui, Y. H., Ed.; VCH Publishers: New York, 1994; pp 57−96.

(12) Soga, T.; Ross, G. A. Simultaneous determination of inorganic anions, organic acids, amino acids and carbohydrates by capillary electrophoresis. *J. Chromatogr. A* **1999**, *837*, 231−239.

(13) López Martínez, N.; Rodrígues Roldán, A. Aplicación de la electroforesis capilar micelar al análisis cuantitativo de ácidos carboxílicos. *Alimentaria* **1993**, *Junio*, 71−74.
(14) Chen, J.; Preston, B. P.; Zimmerman, M. J. Analysis of organic acids in industrial samples. Comparison of capillary electrophoresis and ion chromatography. *J. Chromatogr. A* **1997**, *781*, 205−213.
(15) Swaisgood, H. E. Characteristics of edible fluids of animal origin: milk. In *Food Chemistry*; Fennema, O. R., Ed.; Marcel Dekker Inc: New York, 1985; pp 791−828.
(16) Nelson, R. J.; Burgi, D. S. Temperature Control in Capillary Electrophoresis. In *Handbook of Capillary Electrophoresis*; Landers, J. P., Ed.; CPC Press: London, UK, 1994; pp 549−562.
(17) Fernández-García, E.; McGregor, J. U. Determination of organic acids during the fermentation and cold storage of yogurt. *J. Dairy Sci.* **1994**, *77*, 2934−2939.
(18) Galli, V.; Olmo, N.; Barbas, C. Development and validation of a capillary electrophoresis method for the measurement of short-chain organic acids in natural rubber latex. *J. Chromatogr. A* **2000**, *894*, 135−144.
(19) Foster, S. J.; Johnstone, K. Pulling the trigger: the mechanism of bacterial spore germination. *Mol. Microbiol.* **1990**, *4*, 137−141.
(20) Ortigosa, M.; Torre, P.; Izco, J. M. Effect of pasteurization of ewe's milk and use of a native starter culture on the volatile components and sensory characteristics of Roncal cheese. *J. Dairy Sci.* **2001**, *84*, 1320−1330.

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