Rapid Simultaneous Determination of Organic Acids, Free Amino Acids, and Lactose in Cheese by Capillary Electrophoresis

J. M. Izco, M. Tormo, and R. Jiménez-Flores

Dairy Products Technology Center
California Polytechnic State University
San Luis Obispo, CA 93407

ABSTRACT

A capillary electrophoresis (CE) method for the simultaneous separation of 11 metabolically important organic acids (oxalic, formic, citric, succinic, orotic, uric, acetic, pyruvic, propionic, lactic, and butyric), 10 amino acids (Asp, Glu, Tyr, Gly, Ala, Ser, Leu, Phe, Lys, and Trp), and lactose has been optimized, validated, and tested in dairy products. Repeatability and linearity were calculated for each compound, with detection limit values as low as $0.2 \cdot 10^{-2}$ mM for citric acid and Gly. The method was applied to analyze yogurt and different varieties of commercial cheeses. This method yielded specific CE patterns for different varieties of cheese. Also, it has been shown to be sensitive enough to measure small changes in composition of some of those compounds in fresh cheese stored under accelerated ripening conditions for 2 d at 32°C (e.g., from 1728.3 ± 45.0 to 1166.7 ± 4.5 mg/100 g of DM in the case of lactose, or from 23.5 ± 0.6 to 76.8 ± 16.7 mg/100 g of DM in the case of acetic acid).

(**Key words:** organic acids and amino acids, lactose, cheese, capillary electrophoresis)

Abbreviation key: BGE = background electrolyte, CE = capillary electrophoresis, CTAB = hexadecyltrimethylammonium bromide, EOF = electroosmotic flow, *f* = response factor, i.d. = internal diameter, IS = internal standard, LAB = lactic acid bacteria, NSLAB = non-starter LAB, PDC = 2,6-pyridinedicarboxylic acid, p.s.i. = pound square inch.

INTRODUCTION

Three primary biochemical events occur during manufacturing and ripening of cheese: glycolysis, proteolysis, and lipolysis. These changes together with numer-

ous concomitant secondary transformations taking place during ripening determine the finer aspects of the flavor and texture of cheese. During the manufacturing phase, lactose is converted to lactic acid by the starter bacteria. In the case of Cheddar cheese, most of the lactic acid is produced in the vat before salting and molding, whereas for most other varieties, acidification occurs after the curd has been placed in molds (Fox, 1993).

Organic acids may appear in cheese as a result of hydrolysis of milk fat during lipolysis (FFA such as acetic or butyric), normal bovine biochemical metabolism (citric, orotic, and uric), or bacterial growth (lactic, acetic, pyruvic, propionic, and formic). These are the major products of carbohydrate catabolism of lactic acid bacteria (LAB). The resulting acidity prevents the development of spoilage and pathogenic microorganisms, improving the hygienic quality of cheese. However, 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 (Izco et al., 2002). Quantitative determination of organic acids is important for monitoring bacterial growth and activity and for nutritional reasons and because organic acids contribute to the flavor and aroma characteristics of most cheeses (Fox, 1993; González de Llano et al., 1996).

Proteolysis is the most complex of the three events during ripening. It is also the major contributor to the changes taking place in the cheese matrix and occurs in most pressed-curd cheeses (Irigoyen et al., 2001). Casein breakdown liberates amino acids and peptides that directly contribute to cheese flavor. Because non-starter LAB (NSLAB) possess a wide range of hydrolytic enzymes, they affect proteolysis and lipolysis during ripening and, therefore, the flavor of the cheese (Lane and Fox, 1996). Also, NSLAB adjuncts have been shown to accelerate the production of amino acids (Lynch et al., 1996). Furthermore, some amino acids (e.g., Glu, Leu, and Lys) have been linearly correlated with ripening time in Idiazábal and Ossau-Iraty cheese and are very helpful indicators of the degree of ripening (Izco et al., 2000).

Received March 6, 2002.

Accepted March 21, 2002.

Corresponding author: R. Jiménez-Flores; e-mail: rjimenez@calpoly.edu.

These types of compounds have been analyzed by a variety of different analytical techniques. Until a few years ago, organic acids were commonly analyzed by chromatographic techniques. Most methods developed to analyze organic acids in dairy products are based on HPLC (Fernández-García and McGregor, 1994; Mullin and Emmons, 1997). Amino acids are analyzed by HPLC methods with pre- or postcolumns derivatization and ultraviolet or fluorescence detection (Izco et al., 2000). On the other hand, the analysis of carbohydrates normally is performed by HPLC with a refractive index detector, whereas the analysis of lactose in milk may be carried out by polarimetric, gravimetric or enzymatic methods (AOAC, 1995). Because analytical instrumentation, separation columns, mobile phases, and detectors are different, at least three different systems and methods are necessary for a complete analysis. Therefore, an easy, efficient, and rapid simultaneous analysis method for these compounds would be a great benefit.

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. Recently we developed a CE method to analyze 11 metabolically important organic acids (Izco et al., 2002). The fact that the amino acids and lactose can be charged (using pH values higher than their respective pK_a values would have a net negative charge) suggests that they could be separated by CE. By applying a negative voltage, the 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) inside the capillary and promote the comigration of the analytes with the EOF, thus speeding up the analysis. Since organic acids, amino acids, and lactose have little or no UV absorbance, detection could be accomplished by indirect UV by using a background electrolyte (BGE).

The aim of this work was to establish a CE method for the simultaneous separation and quantification of organic acids, lactose, and several amino acids in cheese and in other dairy products.

MATERIALS AND METHODS

Instrumentation

All the experiments were performed with a P/ACE MDQ Capillary Electrophoresis System (Beckman Coulter, Fullerton, CA) using a photo-diode-array detector. The system was controlled by an IBM computer equipped with 32 Karat Software for data collection and analysis. All 105-cm effective length uncoated fused-silica capillaries (75 μm i.d.) used in this work

were purchased from Bio-Rad Laboratories (Richmond, CA).

Reagents

Oxalic, formic, succinic, orotic, uric, pyruvic, and lactic acids, Asp, Glu, Tyr, Gly, Ala, Ser, Leu, Phe, Lys, Trp, sodium propionate, and lactose monohydrate, were obtained from Sigma (St. Louis, MO); citric, acetic, and butyric acids were purchased from Fisher Scientific (Pittsburgh, PA). Ultrapure water (18.2 M Ω) prepared by treating deionized water with a Barnstead/Thermolyne System (Dubuque, IA) was used to prepare all solutions. Individual stock solutions of each compound at 1000 ppm (10,000 ppm in case of lactose) were prepared by dissolving the proper quantity in 4.5 mM H₂SO₄ containing 15 ppm of boric acid as an internal standard (IS), except orotic and uric acids, Asp, Glu, and Tyr, which were prepared in 0.1 N NaOH. The reagents used to prepare the running buffer were analytical or reagent grade: CTAB was purchased from Sigma and 2,6-pyridinedicarboxylic acid (PDC) was obtained from Aldrich (Milwaukee, WI).

Electrophoretic Procedures and Conditions

The conditions used to analyze the samples were based on the results obtained previously (Izco et al., 2002) with some modifications. The BGE was prepared daily with 20 mM PDC and 0.5 mM CTAB. The pH of the buffer was adjusted at 12.15 with 1 M NaOH. The separations were carried out on fused-silica capillaries with 105 cm of effective length $\times$ 75 μm i.d. Before first use, a new capillary was pretreated with 0.25 M NaOH for 10 min, followed by water for 10 min and BGE for 10 min. Before each run, the capillary was washed for 3 min with 0.25 M NaOH and preconditioned with run electrolyte for 3 min at 60 p.s.i. Before storing it, the capillary was rinsed for 3 min with water and dried by passing air for 1 min at 60 p.s.i.

The sample was injected by hydrodynamic injection for 10 s at 1 p.s.i.. The separation was performed at -25 kV and the capillary was thermostated at 25°C. The wavelength for indirect UV detection was selected at 230 nm, and the signal with negative peaks was inverted to obtain a more familiar electropherogram to integrate and process. However, orotic and uric acids absorb at 230 nm, drastically decreasing the sensitivity for orotic acid, and uric acid appeared as a negative peak. Nevertheless, our CE System is equipped with a photo-diode array detector that allows select different wavelengths. Because these two compounds can be easily detected at 300 nm, we set the detector at both λ = 230 and 300 nm. In this form, for the same analysis we

Figure 1. Electropherograms at $\lambda = 230$ and 300 nm corresponding to the analysis of a standard mixture at 10 ppm (100 ppm in the case of lactose) in 4.5 mM H_2SO_4 containing boric acid as an Internal Standard (15 ppm of IS): 1: sulfuric, 2: oxalic, 3: formic, 4: citric, 5: succinic, 6: orotic, 7: Asp, 8: uric, 9: Glu, 10: acetic, 11: pyruvic, 12: Tyr, 13: Gly, 14: propionic, 15: lactic, 16: IS, 17: Ala, 18: butyric, 19: Ser, 20: Leu, 21: Phe, 22: Lys, 23: Trp, 24: lactose.

obtained one electropherogram at 300 nm to quantify orotic and uric acids and another at 230 nm for the rest of compounds (Figure 1).

Samples and Sample Preparation

Pasteurized whole milk was obtained from the Dairy Products Technology Center (DPTC, San Luis Obispo, CA). All the commercial samples of cheese and plain yogurt were purchased at local stores. For the extraction of the compounds of interest 1 g (200 μl in the case of milk) of sample was weighed and, depending on the commercial sample, a volume of sample buffer consisting of 4.5 mM H_2SO_4 containing 15 ppm of boric acid as IS was added (1 ml in the case of milk, 12 ml in the case of yogurt, 20 ml in the case of fresh cheese, 50 ml in the case of Cheddar cheese, and 100 ml for Parmesan, Roncal, and Blue cheese). The mixture was homogenized for 1 min using an Ultra-Turrax blender and, after that, stirred for 15 min. One milliliter of the sample was centrifuged a $14,000$ rpm for 1 min, and the supernatant was filtered through a 0.45 - μm PVDF membrane (GelmanSciences, Ann Arbor, MI).

RESULTS AND DISCUSSION

Separation Optimization and Validation

Recently (Izco et al., 2002), a CE method was developed in our laboratory to separate the organic acids

mentioned this work. In the cited study, potassium hydrogen phthalate was used as background electrolyte together with 0.27 mM CTAB to prepare a pH 11.2 running buffer. However, at the optimum concentration found for phthalate (4.4 mM), the buffer capacity of the running buffer was poor. Although 20 mM PDC was also tested in the cited work by the authors, this background electrolyte was rejected because the baseline became noisy and wavy. Nevertheless, the CE system utilized in the present work controls with great accuracy and precision the temperature of the capillary during the analysis, noticeably improving the baseline. This has allowed the utilization of 20 mM PDC buffer to test several parameters (run voltage, run temperature, concentration, and pH of the running buffer) until the optimal conditions were determined as indicated in Materials and Methods section. Taking into account the pK_a values of the analytes tested, at the pH selected for the running buffer (pH 12.15), we ensured that all the compounds were ionized, including the amino acids and the lactose. In this form, all the compounds tested were totally separated in less than 35 min (Figure 1). An unknown peak appears in the electropherogram due to the running buffer. This peak (sometimes detected as a jump of the baseline, see Figure 3, below, between peaks 2 and 3) appears only when high pH values running buffers are used, due to the high quantity of NaOH added to adjust the pH of the electrolyte. We have previously detected this anomaly (Izco et al., 2002). Butyric acid and Ala were not totally resolved in commercial samples (Figure 2), and it seems that Ala comigrated with an unknown peak detected in the milk after the IS (Figure 3). Also, other important amino acids such as Pro, Gln, Asn, Val, Met, or His were tested. However, these compounds appeared after the peak corresponding to Ser, and some of them comigrated with a negative unknown peak that appears in dairy samples, making their quantification impossible (see Figure 2). Also, the resolution for some of these amino acids was poorer; therefore, we decided to validate the method for the 10 amino acids selected, which are perfectly separated and identified. Some of the amino acids selected are present in different varieties of cheese, e.g., Glu, Leu, Phe, and Lys have been shown to be major in Ossau Iraty cheese (Izco et al., 2000) with a clear relationship between their concentration and the ripening time.

Tables 1 and 2 show the results obtained in the validation of the analytical technique. The calibration curves were calculated by analyzing in duplicate 5 , 10 , 20 , 50 , and 100 ppm of each compound, except in the case of lactose, which was calculated using 50 , 100 , 200 , 500 , 1000 , 5000 , and $10,000$ ppm in order to cover the normal concentration of lactose in milk (Table 1). Boric acid was used as an IS, and the data points from calibra-

Figure 2. Electropherograms corresponding to a) plain yogurt, b) Farmer's cheese and c) Farmer's cheese stored at 32°C for 2 d. 1: sulfuric, 2: oxalic, 3: formic, 4: citric, 5: phosphate, 6: succinic, 7: uric, 8: acetic, 9: pyruvic, 10: lactic, 11: IS, 12: Ala, 13: butyric, 14: lactose.

tion curves were subjected to a least square regression analysis. The slope (a), intercept (b), and coefficient of determination (r^2) were calculated. Detection limits were estimated as $(3 \times a/b) \times 1/\sqrt{n}$, where a is the inde-

pendent term of the curve, b the slope, and n is the number of replicates. The r^2 values calculated (Table 1) were better than 0.99 for all of the analytes (except for uric acid, 0.9858), showing that the linearity of the method was good and, therefore, suitable for quantification, with detection limits as low as $0.2 \cdot 10^{-2}$ mM in the case of the citric acid and Gly.

The precision of the method expressed as the coefficient of variation for five determinations of a standard mixture at 20 ppm (200 ppm in case of the lactose) was between 0.2 to 1.1% for retention time, except in the case of lactose, which showed lower repeatability of the migration time (Table 1). However, the CV values for peak areas were better than 5% for all of them (including lactose), except oxalic acid (6.4%). That higher CV calculated for oxalic acid could be because this acid migrates close to the big peak of sulfate, which makes its quantification more difficult.

The results obtained are better than those previously obtained in the analysis of organic acids (Izco et al., 2002), where CV values around 10% for uric and citric

acid, and better than 7% for the rest, were calculated. Consequently, the analytical conditions used in this work have clearly improved the CE method. Similar results have been reported by Wu et al. (1995) or Galli et al. (2000), using phosphoric acid as BGE to analyze some of these organic acids in different matrixes, and slightly better than those recorded by Chen et al. (1997), who calculated CV values of 6% using phthalate as electrolyte.

Since 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 (Fernández-García and McGregor, 1994; González de Llano et al., 1996), our proposal was not to validate the method of extraction, but to estimate the accuracy of the analytical technique. Blue cheese was extracted, and the sample obtained was spiked with 10 and 20 ppm of five organic acids (formic, citric, uric, acetic, and lactic) and five amino acids (Asp, Tyr, Leu, Phe, and Trp). Recovery was calculated for both cases (10 and 20 ppm) using the responses of the peaks and the average of the percentage recovery calculated are shown in Table 2. Since no lactose was detected in Blue cheese, cream cheese was used to calculate recovery by spiking lactose at 2500 and 1250 ppm. Recovery was close to 100% for the compounds indicated. These results are similar to the recovery calculated when extracting organic acids from milk, cheese, or yogurt with H_2SO_4 (Fernández-García and McGregor, 1994; González de Llano et al., 1996). Also, by using the high shear blender (Ultra Turrax), we shortened the extraction time from 1 h (González de Llano et al., 1996) to 15 min.

Application for Commercial Dairy Samples

For all of the commercial samples, one extract was prepared from each sample, and duplicate analysis was conducted on each extract. Figure 2 shows the electropherograms corresponding to some of the commercial samples tested. The results obtained are shown in Table 3. Two electropherograms ($\lambda = 230$ nm) corresponding to pasteurized whole milk and milk ripened for 30 min with starter bacteria during manufacture of Cheddar cheese are shown in Figure 3. To simplify the figure, only the electropherogram at $\lambda = 300$ nm corresponding to pasteurized milk have been included (the electropherograms were similar). Besides lactose (not shown in the figure), several organic acids (oxalic, formic, citric, orotic, uric, acetic, pyruvic, propionic, and lactic), three amino acids (Asp, Glu, and Gly), and several unknown peaks can be simultaneously detected and quantified in whole milk by this CE technique. As observed in Figure 3, we detected an increase of acetic and lactic acids produced by the metabolism of starter

Figure 3. Electropherogram corresponding to: (a) pasteurized milk and (b) milk ripened with starter for 30 min during manufacture of Cheddar cheese. 1: sulfuric, 2: oxalic, 3: formic, 4: citric, 5: phosphoric, 6: orotic, 7: Asp, 8: uric, 9: Glu, 10: acetic, 11: pyruvic, 12: Gly, 13: propionic, 14: lactic, 15: IS, 16: butyric, ?: unknown.

Table 1. Regression equations for the calibration curves and results of the analysis of the linearity, detection limits, and precision.

Compound	Range (ppm)	Regr. Equation	R ²	Detec. Limit ¹	CV of RT ²	CV of Area ²
Oxalic acid	10–100	Y = 2.6931X – 0.0490	0.9926	3.9·10 ⁻²	1.1	6.4
Formic acid	5–100	Y = 3.0349X + 0.0011	0.9935	0.7·10 ⁻³	1.0	3.5
Citric acid	5–100	Y = 7.2076X + 0.0486	0.9935	0.2·10 ⁻²	1.0	4.9
Succinic acid	5–100	Y = 5.5146X + 0.0206	0.9968	0.8·10 ⁻²	1.1	2.3
Orotic acid	5–100	Y = 8.4391X + 0.0801	0.9910	2.0·10 ⁻²	1.1	4.6
Asp	5–100	Y = 6.2871X – 0.1398	0.9968	4.7·10 ⁻²	1.0	3.2
Uric acid	5–100	Y = 20.868X + 0.0302	0.9858	0.3·10 ⁻²	0.9	4.8
Glu	5–100	Y = 6.4907X – 0.1274	0.9974	4.2·10 ⁻²	1.0	4.5
Acetic acid	5–50	Y = 3.4031X – 0.0227	0.9947	1.4·10 ⁻²	1.1	3.7
Pyruvic acid	5–50	Y = 2.8641X – 0.0272	0.9989	2.0·10 ⁻²	1.0	3.5
Tyr	5–100	Y = 5.9786X – 0.0764	0.9979	2.7·10 ⁻²	1.0	3.6
Propionic acid	5–100	Y = 3.8588X – 0.0059	0.9972	0.3·10 ⁻²	0.2	3.5
Gly	5–100	Y = 2.8296X – 0.0032	0.9942	0.2·10 ⁻²	1.1	5.0
Lactic acid	5–100	Y = 3.0834X – 0.0131	0.9993	1.5·10 ⁻²	1.1	4.8
Butyric acid	5–100	Y = 4.3620X – 0.1162	0.9915	5.7·10 ⁻²	1.0	4.9
Ala	5–100	Y = 4.1434X + 0.0107	0.9957	0.5·10 ⁻²	0.3	4.0
Ser	5–100	Y = 1.8270X + 0.0452	0.9901	5.3·10 ⁻²	0.2	4.2
Leu	5–100	Y = 3.3045X + 0.0973	0.9945	6.2·10 ⁻²	0.2	2.5
Phe	5–100	Y = 2.8356X + 0.0608	0.9979	4.5·10 ⁻²	0.2	4.0
Lys	5–100	Y = 3.3885X – 0.0545	0.9963	4.4·10 ⁻²	0.2	3.7
Trp	5–100	Y = 12.132X – 0.1282	0.9965	2.2·10 ⁻²	0.3	4.0
Lactose	50–10000	Y = 9.6035X – 0.8006	0.9978	18·10 ⁻²	2.7	3.5

¹Detection limit is in mM.

²CV of retention time (RT) and of corrected peak area are in %. CV calculated from a standard mixture containing 20 ppm (n = 5).

Table 2. Percentage recovery of added organic acids, amino acids and lactose.

Compound	% Recovery ¹
Formic acid	102.3
Citric acid	101.3
Asp	98.9
Uric acid	96.0
Acetic acid	93.5
Tyr	104.0
Lactic acid	101.4
Leu	99.6
Phe	102.4
Trp	97.8
Lactose	105.6

¹Average of recoveries calculated after spiking an extract of blue cheese (cream cheese in the case of lactose) with a standard mixture at 10 and 20 ppm (1250 and 2500 ppm in the case of lactose).

culture bacteria after growing for 30 min by this technique (peaks 10 and 14, respectively).

The electropherogram at $\lambda = 230$ nm obtained when analyzing Blue cheese is shown in Figure 4. Twenty-one compounds were identified. A big peak corresponding to phosphate appears in the electropherogram, making the quantification of succinic acid difficult. Because the concentration of inorganic phosphate in milk is 19 to 23 mM (Walstra et al., 1999), it is logical to find phosphate in cheese. Several small peaks and one big negative peak have been detected, but they could not be identified. On the other hand, there are amino acids appearing between the peaks 18 and 19 that have not

Figure 4. Electropherogram corresponding to Blue cheese. 1: sulfuric, 2: unknown, 3: formic, 4: citric, 5: phosphoric, 6: Asp, 7: uric, 8: Glu, 9: acetic, 10: pyruvic, 11: Tyr, 12: Gly, 13: propionic, 14: lactic, 15: IS, 16: Ala, 17: butyric, 18: Ser, 19: Leu, 20: Phe, 21: Lys, 22: Trp.

been labeled because some of those amino acids (Asn, Val, His, Pro, Met) coeluted as a single peak, and we could not ensure their identification.

Yogurt and fresh cheese (Farmer's cheese) showed fewer number of compounds. Asp and Glu (plus Gly in the case of yogurt) were the only FAA detected in both yogurt and fresh cheese. Because of the efficient carbohydrate (lactose in the case of milk) fermentation by LAB producing lactic acid as the major end product (Gonzalez de Llano, et al., 1996), lactic acid was the

Table 3. Concentration (mg/100 g dry matter) of the organic acids, amino acids, and lactose in some commercial samples.

Comp.	Yogurt	Farmer's cheese	Farmer's cheese 32°C ¹	Blue cheese	Cheddar cheese	Parmesan cheese	Roncal cheese
Oxalic	34.9 ± 0.1 ²	25.3 ± 2.7	38.6 ± 6.0	...	37.0 ± 0.4	...	33.4 ± 0.1
Formic	30.9 ± 0.1	12.0 ± 1.0	12.3 ± 2.1	12.2 ± 0.2	39.4 ± 0.1	9.6 ± 1.0	57.3 ± 21.1
Citric	1386.6 ± 0.7	143.1 ± 2.9	110.4 ± 11.6	71.7 ± 2.0	195.4 ± 8.6	228.2 ± 5.6	...
Succinic	0.2 ± 1.0	...	...	...	...	...	4.3 ± 0.1
Orotic	82.8 ± 0.1	6.0 ± 0.1	4.4 ± 0.3	4.4 ± 0.8	...	...	...
Uric	17.7 ± 0.1	0.5 ± 0.0	0.5 ± 0.1	3.5 ± 2.0	...	...	...
Acetic	64.6 ± 0.4	22.7 ± 0.6	69.8 ± 16.7	24.0 ± 6.3	43.4 ± 1.0	112.0 ± 4.6	239.7 ± 5.9
Pyruvic	32.3 ± 2.1	...	...	73.2 ± 1.7	48.6 ± 0.3	...	...
Propionic	...	...	...	12.6 ± 5.0	...	...	...
Lactic	8230.0 ± 56.4	2488.0 ± 76.2	2217.6 ± 349.8	2691.2 ± 240.4	2185.2 ± 4.0	3143.8 ± 80.5	3615.3 ± 83.2
Butyric	42.0 ± 2.3	...	...	79.2 ± 1.3	89.2 ± 2.4	55.8 ± 1.9	...
Asp	26.8 ± 0.2	11.0 ± 0.1	10.6 ± 0.1	274.5 ± 41.1	70.4 ± 0.3	232.2 ± 5.2	281.0 ± 0.2
Glu	35.9 ± 1.3	15.0 ± 0.1	15.0 ± 0.6	1025.6 ± 15.6	266.9 ± 3.6	1070.5 ± 5.6	700.6 ± 3.4
Tyr	...	...	...	70.6 ± 2.0	56.0 ± 0.7	197.7 ± 3.2	45.5 ± 3.1
Gly	1.0 ± 0.1	...	...	163.5 ± 2.6	27.5 ± 0.5	211.6 ± 4.5	51.6 ± 1.5
Ala	...	...	...	344.8 ± 1.0	256.4 ± 0.0	542.0 ± 5.8	93.1 ± 1.4
Ser	...	...	...	275.8 ± 3.2	50.8 ± 0.1	512.5 ± 8.1	...
Leu	...	...	...	1812.4 ± 10.1	300.5 ± 1.6	1915.9 ± 6.7	646.9 ± 7.2
Phe	...	...	...	515.0 ± 12.1	156.3 ± 4.0	628.5 ± 1.6	238.9 ± 0.4
Lys	...	...	...	1681.4 ± 20.3	158.5 ± 1.9	1669.2 ± 11.2	427.8 ± 23.5
Trp	...	...	...	72.9 ± 2.3	...	52.6 ± 0.1	...
Lactose	42289.2 ± 47.8	2298.7 ± 45.0	1941.7 ± 4.0	...	...	...	...

¹Farmer's cheese stored at 32°C for 2 d.

²Standard deviation of one extract analyzed in duplicate (n = 2).

most abundant organic acid found in yogurt (8230.0 mg/100 g of DM). Concentration of orotic acid found in yogurt (~80 mg/100 g DM) was slightly higher than the values recorded by Fernández-García and McGregor (1994) when using HPLC to quantify organic acids in yogurt. The quantity of orotic acid in milk depends on the cow's origin, diet, and lactation (Gaia et al., 2000). It is an intermediate product in the synthesis of nucleotides and a growth factor for yogurt starter cultures; a decrease of up to 48% in orotic acid content during manufacturing and storage of yogurt has been reported (Arla, 1982). Concentration of citric acid remaining in yogurt (1386.6 mg/100 g of DM) was similar to other results (2.3 mg/g) recorded in the literature (Fernández-García and McGregor, 1994).

Acetic acid is another important organic acid detected in yogurt, probably formed as product of the fermentation of lactose and citric acid. As observed in Table 3, lactose has not been completely consumed by the starter bacteria in yogurt. In the same way, an important quantity remains intact in fresh cheese (2298.7 mg/100 g of DM). To test the sensibility of this method, the same fresh cheese was stored at 32°C for 2 d, and that period was enough to clearly detect a decrease of 15% of lactose and 24% of citric acid, whereas concentration of acetic acid increased approximately threefold. One advantage of the proposed CE method with respect to HPLC is that this method allows simultaneous analysis of lactose and those organic acids in cheese (see Figure 2). In the case of HPLC, those compounds must be analyzed separately to take advantage of the specificity and sensitivity of the detectors, and to avoid problems of coelution of some of them (Mullin and Emmons, 1997).

Lactose was not present in the aged cheese varieties analyzed (Table 3). In fact, ~98% of lactose is removed in the whey as lactose or lactate, and the complete and rapid metabolism of residual lactose remaining in the curd (0.8 to 1.5%) and its component monosaccharides is essential to produce good quality cheese (Fox, 1993).

Lactic acid is the organic acid most abundant in all cheeses tested (Table 3). Acetate may be produced by starter LAB from lactose or lactate or citrate or amino acids and must make some contribution to Cheddar cheese flavor. Nevertheless, the oxidation of lactate to acetate in cheese can be carried out by NSLAB (Fox, 1993). Because Roncal cheese is made with raw milk and the population of NSLAB is abundant, the concentration of acetic acid found in Roncal cheese (239.7 mg/100 g of DM) may be higher than the rest. Acetic acid has been previously detected in Roncal cheese, and the importance of NSLAB for the development of the characteristic flavor of Roncal cheese has been reported also (Ortigosa et al., 2001). Perhaps, this method could be useful for the characterization of cheese made with raw

milk from the same type of cheese, but manufactured with pasteurized milk. In the same form, citrate can be metabolized by starter bacteria to generate acetoin, which is reduced to butan-2,3-diol (diacetyl). In Roncal cheese, these components are reduced to butan-2-one and butan-2-ol by microorganisms present in raw milk. All these compounds are very important flavor components of Roncal cheese (Ortigosa et al., 2001). On the other hand, due to CO₂ production, rapid citrate metabolism can be responsible for the undesirable openness and floating curd in Cheddar cheese. Citric acid is not the first energy source of bacteria, but can be metabolized very rapidly by *Lactococcus lactis* subsp. *diacetylactis* or *Leuconostoc* spp. in Cheddar cheese. Depending on the starter used, citrate can remain constant at 2% (wt/wt) up to 3 mo of ripening, and decrease to 0.1% (wt/wt) at 6 mo (Fox, 1993). Citrate in cheese presumably reflects the concentration of colloidal citrate in milk. The concentration of citric acid in the sample of Cheddar cheese was 195.5 mg/100 g of DM, which is into the normal range (0.2 to 0.5%, wt/wt) of citrate content in Cheddar cheese (Fox et al., 1993).

Proteolysis is the major contributor to the changes taking place in the cheese matrix and occurs in most pressed-curd cheeses. The main proteolytic agents are: the natural proteases of the milk, milk-clotting enzymes retained in the curd, and proteases and peptidases from starter and nonstarter bacteria. However, FAA are released mainly by the action of microbial enzymes from the starter culture (Irigoyen et al., 2001). Soluble Tyr has been used as rapid method to monitor cheese ripening because its concentration is higher in cheeses in which β -casein is extensively hydrolyzed, e.g., Blue, Cheddar, and Parmesan (Marcos and Esteban, 1993). The concentrations of this amino acid in these cheeses were 70.6, 56.0, and 197.7 mg/100 g of DM, respectively, whereas 45.5 mg/100 g of DM was found in Roncal cheese (See Table 3). In fact, the two fractions of β -casein are not extensively hydrolyzed during ripening of Roncal cheese (Irigoyen et al., 2000), perhaps due to higher resistance of ovine β -casein fractions to proteolysis (Izco et al., 1999).

Many of the major amino acids quantified in the cheeses tested have been also reported previously, e.g., Glu, Leu, Phe, Leu, and Lys are some of the major amino acids in Parmigiano Reggiano cheese, a high proteolytic cheese very similar to Parmesan cheese (Battistotti and Corradini, 1993); they are also major in Idiazabal and Ossau-Iraty cheese (Izco et al., 2000). Compared to the proteolysis in other cheeses, that in mold-ripened cheeses (particularly in Blue cheeses) is higher (Gripon, 1993). Extreme proteolysis results in Blue cheese and Parmesan having higher concentration of total free amino acids (measured as the sum of the

free amino acids individually quantified) than the rest of the cheeses.

CONCLUSIONS

A CE procedure has been optimized and found to be well suited to the simultaneous analysis of lactose, the 11 metabolically important organic acids, and the 10 amino acids mentioned. Capillary electrophoresis has been shown to achieve adequate separations, and the suitability of the technique has been verified by the analysis of linearity and precision.

The proposed method appears to be an alternative to other analytical techniques, with the additional advantages of low solvent consumption (milliliters per day vs. liters per day for HPLC), short time of analysis, no hazardous solvents, and low costs compared with others (more than 1000 analysis were run with the same capillary without loss of resolution; in the case of HPLC, columns must be deeply cleaned and regenerated after a few analysis). Nevertheless, the principal advantage is that the great versatility of the method allows analysis of all those compounds simultaneously, whereas to analyze them by HPLC different columns, buffers, and chromatographic systems are necessary. The procedure offers faster and simpler sample preparation for the analysis, e.g., HPLC analysis of amino acids requires precipitation of the protein and pre- or postcolumn derivatization of the previously extracted free amino acids to detect them.

It has been demonstrated that the proposed technique yields different CE patterns that can be used as "reference fingerprints" for cheese varieties. The lactose and organic acid content can be used to monitor the fermentation process, providing additional information about the type of fermentation, whereas the amino acid contents are reliable indices of the proteolysis and therefore, of the ripening of cheese.

ACKNOWLEDGMENTS

The authors are very grateful to the Secretaría de Estado de Educación, Universidades, Investigación y Desarrollo of the Ministry of Education and Culture of Spain, to California Dairy Research Foundation, and to the California Agricultural Research Initiative for the funding provided for this study.

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