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New Capillary Electrophoresis Method for the Determination of Furosine in Dairy Products

BELINDA VALLEJO-CORDOBA,* MIGUEL A. MAZORRA-MANZANO, AND AARÓN F. GONZÁLEZ-CÓRDOVA

Centro de Investigación en Alimentación y Desarrollo, A.C., Carretera a la Victoria Km 0.6, P.O. Box 1735, Hermosillo Sonora, 83000 Mexico

A new capillary electrophoresis (CE) method was established for the quantitative determination of furosine in dairy products. Sample preparation and suitable electrophoretic conditions allowed accurate and reproducible quantitation of furosine in dairy products. Sample preparation consisted of drying hydrolyzed samples, redissolving them in 0.2 M NaOH, and purifying them by solid-phase extraction. The electrophoretic separation was carried out in an uncoated capillary maintained at 30 °C using 0.1 M phospate buffer containing the additive hexadecyl trimethylammonium bromide (HDTAB, 1.2 mM) (pH 7.0) under 10 kV voltage and reverse polarity. Coefficients of variation of less than 2.25% for migration time and 5.80% for peak areas indicated that the technique was reproducible. The calibration curve followed a linear relationship with a highly significant ($p < 0.01$) coefficient of multiple determination ($R^2 = 0.997$). The limit of quantitation was 0.5 ppm, a concentration that corresponds to 4.5 mg/100 g of protein in milk samples. Furosine concentration (mg/100 g of protein) ranges of different dairy products (raw, pasteurized, UHT, and evaporated milks and yogurt) agreed with ranges previously reported. Therefore, the CE method presented is a suitable technique for the routine assessment of furosine in dairy products.

KEYWORDS: Capillary electrophoresis; furosine; dairy products

INTRODUCTION

Furosine (*E-N*-(2-furoylmethyl)-L-lysine) is an amino acid derivative formed during the acid hydrolysis of such Amadori products as fructose lysine, lactulose lysine, and maltulose lysine, which are generated in the early stages of the Maillard reaction during the heat processing of foods (*1*). For this reason, estimates of the extent of protein damage caused by heating in the first stages of that reaction are often based on determinations of the amount of furosine that forms during the acid hydrolysis of foods (*1*). The Maillard reaction, which is known as the nonenzymatic reaction between reducing sugars and primary amino groups, is a very complex set of reactions that take place during the processing and storage of most foods and cause deterioration of their nutritive value (*2*, *3*).

In dairy products, even in the absence of browning or off-flavors, their nutritive value may be considerably reduced due to the formation of the protein-bound Amadori product (lactulosyl lysine) formed by the reaction of lactose and the ϵ-amino groups of lysine in the first stage of the Maillard reaction, almost selectively blocking the lysine residues during industrial treatments of milk (*2*). Milk is very rich in lysine, and, due to the lactose content, furosine rises quickly with increasing processing time/temperature conditions. Thus, furosine can be considered as an indicator of the extent of the early Maillard reaction related to the type and intensity of the food processing conditions, as well as to the storage conditions, and can be used as a suitable indicator of the quality of dairy products *(4)*. Furosine can be used as an indicator of the heat damage of milk and for the distinction of UHT milk, pasteurized milk, and in-container sterilized milk (*4*). Moreover, on the basis of the furosine value, it was possible to identify the presence of reconstituted milk powder in raw or pasteurized milk (*5*).

Several techniques have been described for furosine analysis: (a) GC after derivatization to heptafluorobutyryl isobutyl esters, (b) amino acid analysis, (c) HPLC, and (d) capillary zone electrophoresis (*2*, *6*). Ion-pair reverse-phase high performance liquid chromatography (HPLC) appears to be the most popular technique for the determination of furosine in dairy products (*7*).

As capillary electrophoresis (CE) is an attractive analytical technique because of its speed of analysis, ease of method development, and saving of solvent, a CE method was the subject of a preliminary study (*8*). This method was found to be suitable in terms of repeatability, efficiency, and sensitivity but was inadequate in terms of accuracy when low levels of furosine were determined. Thus, an improved CE separation of furosine was reported with reverse polarity; such an approach allowed very efficient separation, but reliable quantitative data were obtained only for standard solutions of furosine (*6*). Thus,

* Author to whom correspondence should be addressed [telephone 52-62-80-00-57; fax 52-62-80-04-21; e-mail vallejo@cascabel.ciad.mx].

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a different CE method was reported by using a capillary with an extended-path-length detection cell, to increase sensitivity, and 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid solution at pH 7.0 as the run buffer. Although this method reported good sensitivity and accuracy, a slight tailing of the furosine peak was still noticed (*6*). In addition, the use of a specific capillary with an extended-path-length detection cell, instead of a regular capillary, was required.

Thus, in this work, a new reproducible, accurate, and sensitive capillary electrophoresis method for determining furosine in milk and dairy products is presented.

MATERIALS AND METHODS

Samples. Raw milk samples were obtained from five different local dairy farms. Pasteurized (10 samples), UHT (10 samples), evaporated milk (2 samples), and yogurt (2 samples) were from different brands collected in the local market.

Reagents. Hexadecyl trimethylammonium bromide (HDTAB), methanol, HPLC grade acetonitrile, sodium hydroxide, phosphoric and formic acid, and sodium dihydrogen phosphate monohydrate were from Sigma (St. Louis, MO). Hydrochloride acid was from EM Science (Gibbstown, NJ). Pure (>99%) crystalline furosine dihydrochloride was obtained from Neosystem Laboratories (Strasburg, France).

Run Buffer. The run buffer was 0.1 M phosphate at pH 7.0 containing 1.2 mM HDTAB.

Sample Preparation. Samples were prepared as was previously reported in ref *7* with modifications. Samples containing 15−20 mg of protein or 0.5 mL of milk were hydrolyzed with 3 mL of 7 N HCl under nitrogen at 110 °C for 24 h in a screw-cap Pyrex vial. After hydrolysis, samples were protected from light exposure. The hydrolyzed sample was dried under vacuum at 65 °C for 10 min in a rotary evaporator. The dried sample was reconstituted in 1 mL of 0.2 M NaOH and filtered with a medium-grade filter (0.22 μm). A portion (0.5 mL) of the filtrate was purified using a Sep-pack C_{18} cartridge 600 mg (Alltech Associates, Inc., Deerfield, IL). The cartridges were prewetted with 5 mL of methanol and 10 mL of water before use. Furosine elution was carried out using a 1.5 mL solution of water:acetonitrile:formic acid (94.8:5.0:0.2); the first 0.5 mL of elution solution was discarded, and the remaining 1 mL containing furosine was collected for CE analysis.

Capillary Electrophoresis. Capillary free zone electrophoresis was performed using a Beckman P/ACE 2000 system (Beckman Coulter, Inc., Fullerton, CA) provided with UV detection at 280 nm. A bare silica capillary with an internal diameter of 50 μm, 37 cm total length, and 30 cm effective length from Bio-Rad (Bio-Rad Laboratories, Hercules, CA) was used. Every working day the capillary was conditioned by first flushing with 1 M NaOH for 15 min, then washed with distilled deionized water for 10 min, and finally rinsed with run buffer for 5 min. Between runs, the capillary was rinsed with 0.1 M NaOH for 2 min, followed by water for 2 min and run buffer for 2 min. Samples were injected with pressure (0.5 psi) for 10 s. A voltage of 10 kV with reverse polarity was applied during 10 min, and the capillary temperature was maintained at 30 °C. Data were collected by a System Gold Software data system (Beckman Coulter, Fullerton, CA).

Furosine Quantitation by Capillary Electrophoresis. A calibration curve was constructed by preparing standard solutions of 1, 2, 5, 10, 20, 35, and 50 ppm of furosine in distilled deionized water. Analysis was carried out in triplicate, and furosine was expressed as mg/100 g of protein. Protein content was determined according to the AOAC procedure (*9*). Recovery studies were performed by spiking samples with 2−50 ppm of furosine.

RESULTS AND DISCUSSION

A critical factor that determined the accuracy and repeatability of the method was the pH of the sample. Adjusting the pH of the sample to 7.0, by redissolving the dried sample hydrolysate in 1 mL of 0.2 M NaOH, before the purification step was a critical factor because it determined good symmetry of the furosine peak. Typical electropherograms of a standard solution of furosine, different milk types, and yogurt showed very good peak symmetry for the furosine peak under the conditions used in this study (**Figures 1−5**). It is known that acid-hydrolyzed samples cannot be directly injected into the CE system mainly because of its high ionic strength and low furosine content (*6*). A previously reported CE method (*8*) presented the problem of asymmetrical peak shape and subestimation of furosine. The tailing of the furosine peak was assumed to be due to the difference in initial pH values in the capillary between the running buffer zone (pH 7.0) and the sample zone (pH 2.0) (*9*). In addition, the hydrolysate was not subjected to SPE purification (*8*). Others (*6*) improved the CE method by including sample purification by SPE followed by drying and redissolving in buffer solution at pH 7.0. However, redissolving the hydrolyzed sample in buffer solution at pH 7.0 may not have been a high enough pH to reduce the difference between the running buffer (pH 7.0) and the sample zone (pH 2.0) because tailing of the furosine peak was still noticed (*6*). Sample composition varies widely depending on the content of carbohydrates, protein, and fat; thus, sample purification by SPE allows many potentially interfering substances to be removed (*6*). In the present method, the hydrolyzed sample was first dried, then redissolved in 0.2 M NaOH to bring the pH to 7.0, and purified through SPE. Thus, there was not a pH difference between the running buffer and the sample zone, and, consequently, the problem of a tailing furosine peak was not observed (**Figures 1−5**).

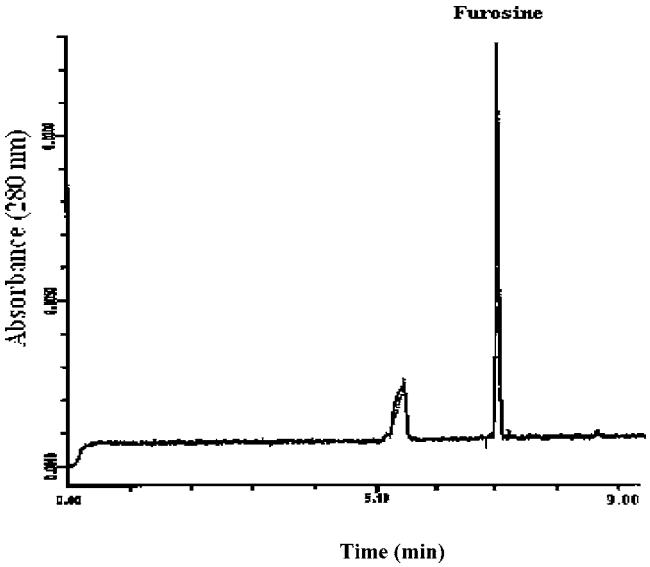

Figure 1. Typical electropherogram of a 50 ppm furosine standard solution.

The use of the additive HDTAB in the run buffer and the operation of the CE under reverse polarity permitted complete separation between the furosine peak and other peaks (**Figures 1−5**). The surfactant HDTAB reverses the charge of the capillary wall to reduce adsorption. Charge reversal is accomplished by ion-pair formation between the cationic headgroup of the surfactant and the anionic silanol group. The hydrophobic surfactant tail extending into the bulk solution cannot be solvated by water. Its solvation need is satisfied by binding the tail of another surfactant molecule. As a result, the cationic headgroup of the second surfactant molecule is in contact with the bulk solution. The capillary wall behaves with cationic character because of this treatment, and the electrosmotic flow is directed toward the positive electrode. As a result,

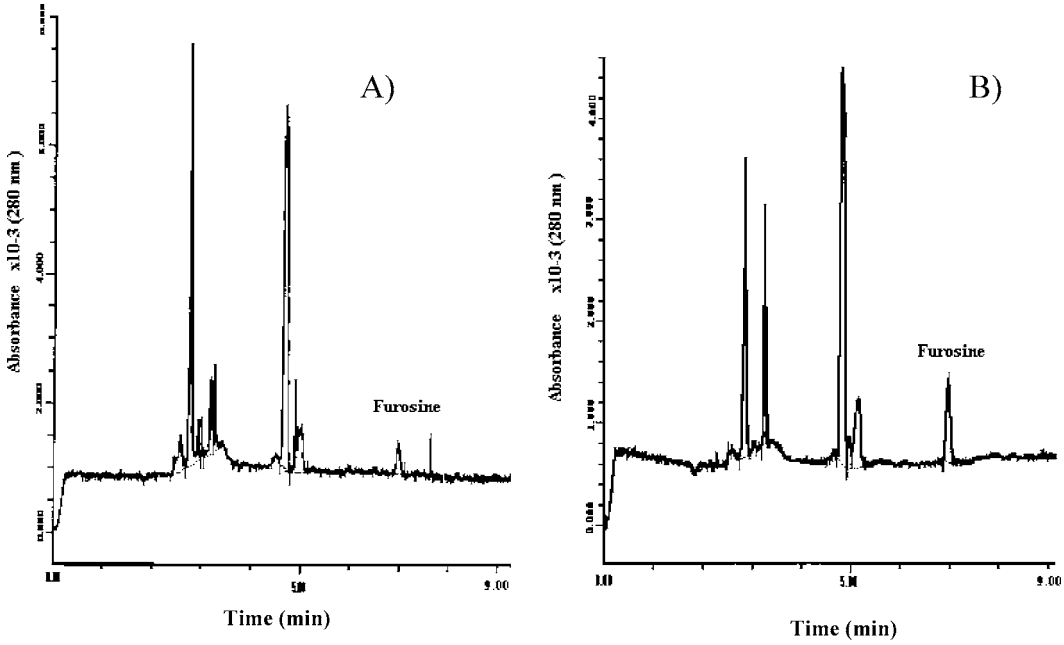

Figure 2. Electropherograms of commercial pasteurized milk samples: (**A**) typical pasteurized milk containing 6.0 mg of furosine/100 g of protein; (**B**) pasteurized milk that may have addition of dry milk, containing 20 mg of furosine/100 g of protein.

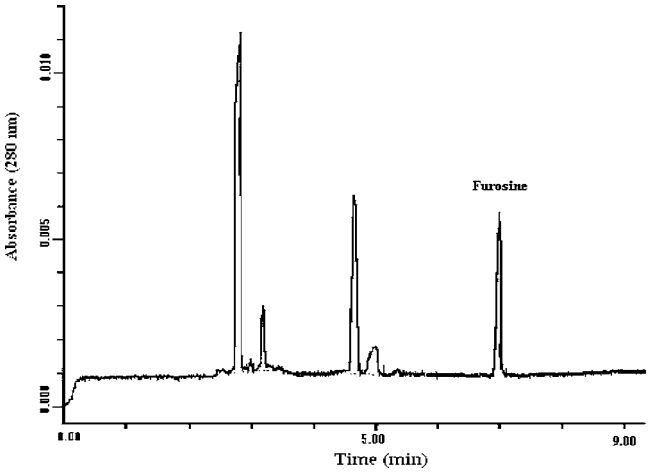

Figure 3. Typical electropherogram of UHT milk containing 269 mg of furosine/100 g of protein.

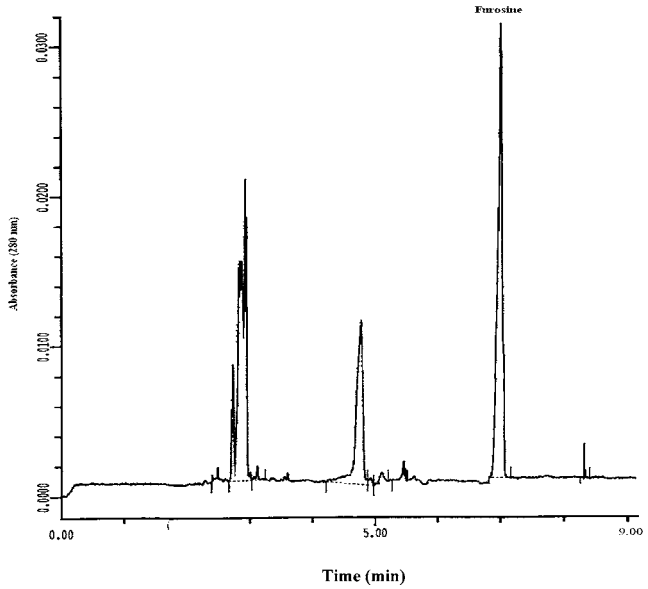

Figure 4. Typical electropherogram of evaporated milk containing 730 mg of furosine/100 g of protein.

it is necessary to operate the CE instrument with reverse (sample-side negative) polarity (*10*).

For accurate quantitation, it was necessary to achieve high reproducibility in migration times and peak areas. Means, standard deviations and coefficients of variation for migration times, and peak areas for the furosine peak of a standard and a milk sample are summarized in **Table 1**. Very good reproducibility of migration times and peak areas was observed with coefficients of variation of less than 2.25% and 5.8%, respectively (**Table 1**), although slightly higher coefficients of variation were observed for the analytical standard. The technique showed very high correlation between furosine concentration and peak areas. The calibration curve constructed ($Y = 0.0802X + 0.0529$) followed a linear relationship with a highly significant ($p < 0.01$) coefficient of multiple determination ($R^2 = 0.997$), where Y was the furosine peak area and X was the standard concentration (ppm). Thus, this calibration curve was used for determining furosine concentrations in different dairy products. Considering a signal-to-noise ratio of 6, the minimum quantifiable amount of furosine was calculated to be 0.5 ppm, a value that corresponds to 4.5 mg/100 g of protein in milk samples. Thus, the sensitivity of the method was comparable to that obtained with the most popular ion-pair reversed-phase (IP-RP) HPLC method (*7*). The method was accurate because furosine recoveries in the different samples ranged from 97% to 109%.

Table 2 shows the furosine concentration ranges of different dairy products. As expected, the lowest furosine concentrations were presented by an unheated product (raw milk) and a mildly heat-treated product (pasteurized milk) (**Table 2**). These concentrations were within the ranges reported for similar products (*3*). However, a concentration of 20 mg of furosine/100 g of protein found in a pasteurized commercial milk sample was not typical and may indicate the addition of dry milk (**Figure 2B**).

The highest furosine concentrations were found for evaporated milk because it is the most severely heat-treated product (**Table 2**). Furosine concentrations for the UHT milks analyzed were

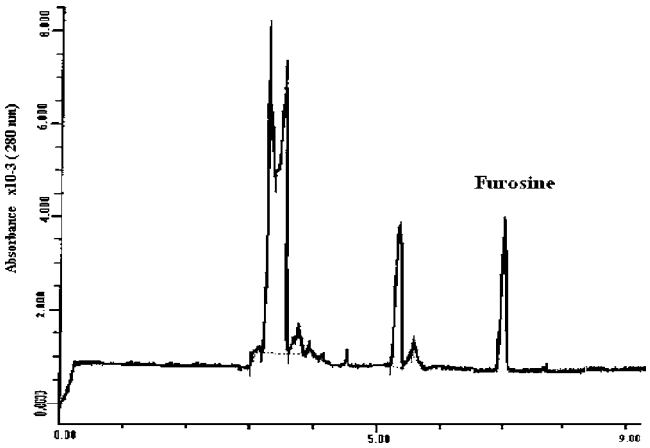

Figure 5. Typical electropherogram of yogurt containing 180 mg of furosine/100 g of protein.

Table 1. Migration Times and Peak Areas Observed in Capillary Electrophoresis Electropherograms of a Furosine Standard and a Milk Sample ($n = 5$)

sample	mean area ± SD[a] (CV, %)[b]	mean migration time ± SD (CV, %)
furosine standard	0.340 ± 0.020 (5.80)	6.57 ± 0.148 (2.25)
milk	0.226 ± 0.0093 (4.13)	6.87 ± 0.023 (0.33)

[a] SD, standard deviation. [b] CV, coefficient of variation.

within the ranges reported for indirect UHT milk, because direct UHT milks present lower concentrations (48.7−74.6 mg/100 g of protein) (*8*). The high values of furosine found for yogurt were probably due to the addition of dry milk that is regularly used in its manufacture (**Table 2**).

In conclusion, the sample preparation and electrophoretic conditions presented in this work provided a capillary electrophoresis method for the quantitative determination of furosine in dairy products. The accuracy and reproducibility of the technique allowed the quantitation of low levels of furosine present in milk. Therefore, the CE method presented is a suitable technique for the routine assessment of furosine in dairy products.

Table 2. Furosine Concentration Ranges (Milligrams per 100 g of Protein) in Different Dairy Products Determined by Capillary Electrophoresis

sample	no. of samples	furosine (mg/100 g of protein)
raw milk	5	ND[a]–4.96
pasteurized	10	4.51–9.04
UHT	10	169–269
evaporated milk	2	730–836
yogurt	2	180–193

[a] ND, not determined, below the limit of quantitation (4.5 mg/100 g of protein).

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