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Improved method for the simultaneous determination of whey proteins, caseins and *para*-κ-casein in milk and dairy products by capillary electrophoresis ☆

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Abstract

A capillary electrophoresis method for the simultaneous determination of whey proteins, caseins and their degradation products, such as *para*-κ-casein, was proposed. The effect of several parameters (pH, ionic strength and concentration of urea in the electrophoresis buffer and applied voltage) on the analysis time and on the separation efficiency of the major milk proteins was studied. Using a hydrophilically coated capillary, in combination with electrophoresis buffer 0.48 *M* citric acid–13.6 m*M* citrate–4.8 *M* urea at pH 2.3, and a separation voltage of 25 kV, a complete separation of β-lactoglobulin and *para*-κ-casein was achieved, permitting the quantification of both components. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Milk; Food analysis; Cheese; Proteins; Whey proteins; Caseins; Lactoglobulin

1. Introduction

In the last few years, the potential of CE, in its different modes, for the rapid separation and measurement of milk proteins has been widely demonstrated [1–4]. Few studies, so far, have investigated proteolysis in milk or cheese applying capillary zone electrophoresis (CZE) [5–7] and micellar electrokinetic capillary chromatography (MECC) [8,9]. Several of the CZE techniques [5–7,10] were based on the method described by De Jong et al. [3], slightly modified by Recio and Olieman [11], which uses methylhydroxyethyl cellulose and pH 3. Although this method allows to study the proteolysis of caseins and cheese, under these conditions, *para*-κ-casein (*para*-κ-CN) co-migrates with β-lactoglobulin (β-Lg), precluding its quantification [12]. Since *para*-κ-CN is a main degradation product from κ-CN by the action of rennet [13] and other proteolytic enzymes from psychrotrophic bacteria [14], its accurate quantification would be of great importance

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to determine the degree of proteolytic degradation in milk and milk products.

CE has recently been used for the characterization of cheeses from different species [15] and processed cheeses [16]. The presence of intact κ-CN can be used to detect and determine the percentage of acid casein and caseinates added as adulterant ingredients in processed cheeses [16]. Nevertheless, cheeses produced with acid coagulation, like Quarg and sour milk cheeses, as Harzer and Mainzer cheese or acid settled Mascarpone, do also contain intact κ-CN, although no acid casein has been added. These restrictions limit the practical application of the methods based on the quantification of intact κ-CN. Furthermore, when processed cheeses are adulterated by the addition of rennet casein, the latter methods cannot be applied. In a further experimental attempt, quantification of rennet casein/caseinate addition could be assayed by measuring the increase of the *para*-κ-CN area.

The aim of this work was to develop a rapid capillary electrophoresis method enabling the simultaneous separation and quantification of denatured β-Lg and *para*-κ-CN. The effect of several parameters, such as pH, concentration of urea and citric acid in the running buffer and voltage applied, on the analysis time and on the separation of the major milk protein was studied.

2. Experimental conditions

2.1. Samples

A model sample was prepared by mixing 18 mg of rennet casein and 300 μl of commercial pasteurized milk. Rennet casein was prepared by incubating milk with chymosin (1:10 000, 0.05 g/l, Chr. Hansen Lacta, E-28028 Madrid, Spain) at 37°C for 30 min. The formed curd was centrifuged and the pellet lyophilized.

β-Lg was purchased from Sigma (St. Louis, MO, USA), *para*-κ-CN was obtained from κ-CN isolated from milk by the method of Zittle and Custer [17] by the following procedure: a 0.5% solution of κ-CN was incubated with chymosin under the same conditions as given above, the reaction was finally stopped by adjusting to pH 9.5 with dilute sodium hydroxide and heating for 15 min at 60°C. After readjusting the pH to 4.6 and centrifugation at 4500 *g* for 15 min, the precipitate was washed three times with 1 *M* sodium acetate buffer pH 4.6. Pure *para*-κ-CN was obtained after exhaustive dialysis and lyophilization. Solutions of each 10 mg/ml of β-Lg and *para*-κ-CN in sample buffer were prepared and added to an isoelectric casein solution at six different final concentrations (0.1–2.5 mg/ml), suitable for a calibration curve. The actual protein contents of β-Lg and *para*-κ-CN determined by elemental analysis were 91.0 and 84.4%, respectively.

Samples of processed cheeses provided through the SMT 4-CT97-2208 project were prepared as described in Ref. [16].

2.2. Capillary electrophoresis

CE was carried out using a Beckman P/ACE System MDQ controlled by its software data system (Beckman Instruments, Fullerton, CA, USA). The separations were performed using a hydrophilically-coated fused-silica capillary column CElect P1, (Supelco, Bellefonte, PA, USA), 600 mm×50 μm I.D., with a slit opening of 100×800 μm. The distance between detection window and outlet was 100 mm, resulting in an effective capillary length of 500 mm. The separations were conducted at 25 or 30 kV grounded at the detector side. Sample solutions were injected for 9 s at 3.4 KPa. Detection was performed on the column at 214 nm. Before each separation the capillary was flushed in the reverse direction with the electrophoresis buffer for 6 min.

Sample buffer (pH 8.5±0.1) consisted of 167 m*M* Tris(hydroxymethyl)aminomethane (reagent grade from Sigma), 42 m*M* 3-morpholino-propanesulfonic acid (BioChemica MicroSelect; Fluka, Buchs, Switzerland), 67 m*M* ethylenedinitrilotetraacetic acid disodium salt (Titriplex III; Merck, Darmstadt, Germany), 17 m*M* DL-dithiothreitol (DTT) (Sigma), 8 *M* urea (Sigma), and 0.5 g/l methylhydroxyethyl cellulose 30000 (Serva, Heidelberg, Germany). Sample buffer (700 μl) were added to 300 μl of model sample. β-Lg, *para*-κ-CN and isoelectric caseins were dissolved in diluted sample buffer (sample buffer–water, 1.5:1, v/v).

The electrophoresis buffer consisted of a solution of citric acid and trisodium citrate (Sigma) in urea

Table 1
Electrophoresis buffers used in the different assays

Assay	Final urea concentration (*M*)	Citric acid–citrate concentration	pH
1	6	0.19 *M*–20 m*M*	3.0±0.05
2	6	0.32 *M*–10 m*M*	2.6±0.05
3	6	0.48 *M*–13.6 m*M*	2.4±0.05
4	4.8	0.48 *M*–13.6 m*M*	2.3±0.05
5	3.4	0.48 *M*–13.6 m*M*	2.2±0.05
6	4.8	0.38 *M*–9.5 m*M*	2.3±0.05
7	4.8	0.32 *M*–13.6 m*M*	2.4±0.05

solution. The buffers assayed are listed in Table 1. Before use, electrophoresis buffer was filtered through a 0.45 μm filter [Millex-HV hydrophilic polyvinylidene difluoride (PVDF), Millipore, Bedford, MA, USA].

2.3. Statistical methods

Linear regression analyses were established using Microsoft Excel 7.0. The determination signal and determination limit were calculated using the Detarchi program, as described by Ortiz and Sarabia [18].

3. Results and discussion

3.1. Selection of analysis conditions

As a starting point, the analysis of the milk proteins of a model sample prepared with rennet casein and pasteurized milk was carried out using an electrophoresis buffer containing 0.19 *M* citric acid in 6 *M* urea at pH 3, according to the method of Recio and Olieman [11]. Under these conditions *para*-κ-CN co-migrates with β-Lg (Fig. 1a). By lowering the pH, increasing the citric acid concentration of the separation buffer, an improved separation of β-Lg and *para*-κ-CN was observed. On the other hand, the distance between κ-CN and β-CN B decreased (Fig. 1b and c). This lower pH promoted the charge difference between β-Lg and *para*-κ-CN, whereas the charge difference of κ-CN and β-CN B decreased. As expected, the total analysis time was slightly increased. The increase in separation time at lower pH is explained by the compression of the ionic double layer due to increased ionic strength. Under these new conditions β-Lg and *para*-κ-CN were effectively separated, which made their quantification possible.

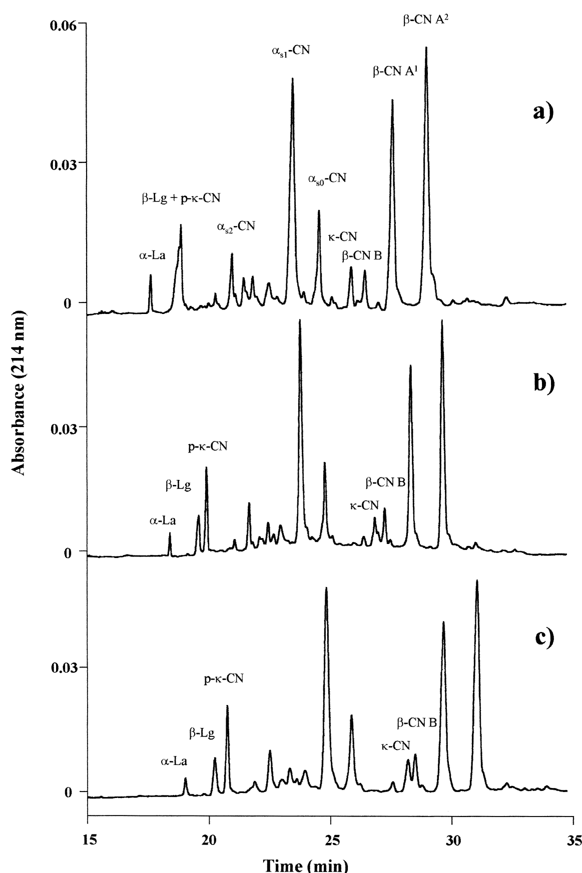

Fig. 1. Electropherograms of a model sample prepared mixing 18 mg of rennet casein and 300 μl of pasteurized bovine milk analysed with an electrophoresis buffer 6 *M* urea and different concentrations of citric acid–trisodium citrate: (a) 0.19 *M* citric acid–20 m*M* trisodium citrate, pH 3.0±0.05; (b) 0.32 *M* citric acid–10 m*M* trisodium citrate, pH 2.6±0.05, and (c) 0.48 *M* citric acid–13.6 m*M* trisodium citrate, pH 2.4±0.05. Separation voltage 25 kV.

To evaluate the peak separation efficiency between β-Lg and *para*-κ-CN, their resolution coefficient was calculated, expressed as the quotient of the difference of migration time of β-Lg and *para*-κ-CN divided by the average of the base width of both peaks. Separation between β-Lg and *para*-κ-CN at

pH 2.4 showed higher resolution (1.34) than at pH 2.6 (1.17).

In order to improve the method, further parameters were modified, maintaining this low pH but trying to reduce analysis time. Viscosity is related to the migration time of solutes because it affects the velocity of the fluid in the capillary, according to Hagen–Poiseuille's equation. Thus, decreasing the urea concentration in the electrophoresis buffer, was used to decrease its viscosity. A buffer prepared with 4.8 *M* urea, with identical citrate composition, indicated shorter migration times. But also the resolution (1.72) between *para*-κ-CN and β-Lg was considerably improved, although β-CN B and κ-CN migrated together. (Fig. 2a and b). This new buffer had a pH of 2.3, showing that this lower urea concentration permitted to reach a slightly lower pH. Further decrease in urea concentration (3.4 *M*) improved the separation of β-Lg and *para*-κ-CN (resolution of 2.4) but had a negative effect on the resolution of the α_{s1}-CN and α_{s0}-CN peaks. These peaks became smaller when compared to the peaks obtained with the former analysis conditions and showed tailing shapes (Fig. 2c). This fact, could be due to α_{s1}-CN and α_{s0}-CN precipitation when the concentration of urea is decreased to 3.3 *M* [19]. Hence, in order to avoid plugging of the capillary columns, the urea concentration was kept at 4.8 M.

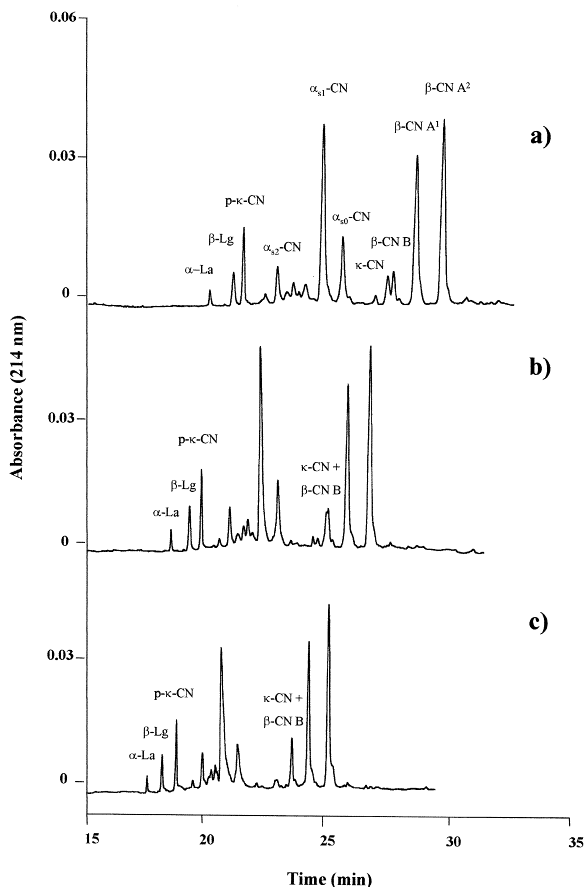

Fig. 2. Electropherograms of a model sample prepared by addition of 18 mg of rennet casein and 300 μl of pasteurized bovine milk analysed with an electrophoresis buffer 0.48 *M* citric acid–13.6 m*M* trisodium citrate and different concentrations of urea. (a) 6 *M* urea, pH 2.4±0.05; (b) 4.8 *M* urea, pH 2.3±0.05; and (c) 3.4 *M* urea, pH 2.2±0.05. Separation voltage 25 kV.

This lower concentration of urea should allow to lower the pH by decreasing citric acid/citrate concentration in the buffer and therefore reduce ionic strength with the consequence of shorter analysis time. However, when trying buffers with 0.38 and 0.32 *M* citric acid, a negative effect on the separation of *para*-κ-CN and β-Lg was observed, showing a decrease of the resolution coefficient to 1.29 and 1.14, respectively. Therefore, the optimal concentration of citric acid in the separation buffer appeared to be at 0.48 *M* (data not shown).

A second alternative to reduce the analysis time was to increase the electric field strength. When a voltage of 30 kV instead of 25 kV was tried, fairly good separation of all milk proteins including *para*-κ-CN was achieved in a 5 min shorter separation time (data not shown). However, the resolution between *para*-κ-CN and β-Lg decreased, although to a still acceptable value (1.29). Depending on the characteristics of the sample and desired analysis time, a compromise had to be taken. We chose 25 kV because it provided the maximum resolution, which can be important in the case of analysis of milk samples containing high amounts of denatured β-Lg, such as UHT milk. The electropherograms of denatured β-Lg show wider peaks, probably due to its lactosylation [20].

3.2. Quantitative analysis

The conditions chosen for quantitative analysis were an electrophoresis buffer which contained 0.48 *M* citric acid–13.6 m*M* trisodium citrate in 4.8 *M*

Table 2
Calibration curves of β-Lg and *para*-κ-CN considering corrected peak area versus concentration in a mixture of these proteins with an isoelectric casein solution[a]

Protein	Slope	Intercept	Correlation coefficient	SEE*
β-Lg	4299±70.77	256±77.54	0.999	180.48
para-κ-CN	3572±56.50	64±54.42	0.999	133.65

[a] The conditions for the CE analysis were: electrophoresis buffer 4.8 *M* urea, 0.48 M–13.6 m*M* citric acid–trisodium citrate at pH 2.3±0.05 and 25 kV.

* Standard error of estimate.

urea (pH 2.3±0.05) and a voltage of 25 kV. Calibration curves for β-Lg and *para*-κ-casein, mixed with an isoelectric casein solution, were calculated. For this purpose, concentrations of β-Lg in the range 0.09–2.28 mg/ml and of *para*-κ-casein in the range 0.08–2.11 mg/ml were analyzed. Plots of velocity-corrected peak area [(peak area×distance between capillary inlet and detection window in mm)/migration time] versus concentration in mg/ml demonstrated that the regression analysis was linear in this range of concentration (Table 2).

Under these separation conditions, determination limits of 0.16 and 0.14 mg/ml and determination signals of 612.1 and 328.3 peak area units for β-Lg and *para*-κ-CN, respectively, were obtained. The mean accuracies were 99.54 and 105.18% for quantitation of β-Lg and *para*-κ-CN, respectively.

In order to check whether the determination of *para*-κ-CN and β-Lg in milk samples was hampered by adsorption to the capillary wall, analysis of a casein solution was performed before and after addition of different amounts of β-Lg and *para*-κ-CN. Table 3 shows acceptable recoveries which demonstrates that adsorption does not significantly affect the quantification.

Table 3
Recoveries for different proteins calculated from the analysis of a solution of casein with added β-Lg and *para*-κ-CN solution using the following conditions: electrophoresis buffer 4.8 *M* urea, 0.48 M–13.6 m*M* citric acid–trisodium citrate at pH 2.3±0.05 and 25 kV

Protein	Added (mg/ml)	Found (mg/ml)	Recovery (%)
β-Lg	0.50	0.42	84
β-Lg	1.00	1.04	103
para-κ-CN	0.50	0.43	86
para-κ-CN	1.00	1.10	110

The repeatability of the method was studied by four consecutive injections of the model sample. The relative standard deviation values (RSDs) of the migration times of β-Lg and *para*-κ-CN were less than 0.3%. For the corrected peak areas, RSD values of 2.5 and 1.3% were achieved for β-Lg and *para*-κ-CN, respectively. The day-to-day variation was assayed by injecting the model sample four different days. The RSD values obtained for the migration times were of 1.7% for β-Lg, and 1.6% in the case of *para*-κ-CN. For the corrected peak areas the values obtained for RSD were 2.8 and 1.3% for β-Lg and *para*-κ-CN, respectively.

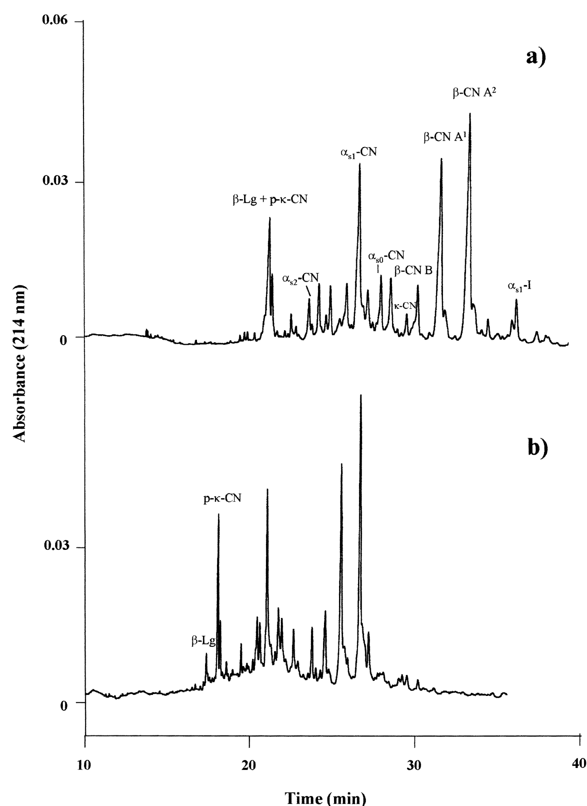

Fig. 3. Electropherograms of a processed cheese analysed with an electrophoresis buffer containing: (a) 6 *M* urea, 0.19 *M* citric acid–20 m*M* trisodium citrate, pH 3.0±0.05; (b) 4.8 *M* urea and 0.48 *M* citric acid–13.6 m*M* trisodium citrate, pH 2.3±0.05. Separation voltage 25 kV.

3.3. Analysis of processed cheeses

Fig. 3 shows the electrophoretic profiles of the casein fraction obtained from a processed cheese, analyzed by using the conditions of assay 1 and 4 as given in Table 1. Under the conditions of assay 1 (0.19 *M* citric acid–20 m*M* citrate–6 *M* urea, pH 3.0±0.05) a double peak with a shoulder at 21 min was observed (Fig. 3a). In order to identify and quantify the contribution of β-Lg and *para*-κ-CN to this peak, the conditions of assay 4 were applied as they provided better resolution between these peaks than assay 3 and a shorter analysis time (Fig. 3b). Under these new conditions, denatured β-Lg was well resolved from the peak corresponding to *para*-κ-CN, making it possible therefore to quantify β-Lg and *para*-κ-CN more accurately.

4. Conclusions

The present results show that when using an acidic capillary electrophoresis separation, a decrease in the pH of the separation buffer (pH 2.4) led to improved resolution between β-Lg and *para*-κ-CN but increased separation time, while a lower urea concentration resulted in a higher resolution and shorter separation time. Additionally, a running buffer containing 3.4 *M* urea led to better separation of β-Lg and *para*-κ-CN but reduced the separation of intact κ-CN from other components as well as the resolution of the α_s-casein group. By using an electrophoresis buffer consisting of 0.48 *M* citric acid–13.6 m*M* trisodium citrate in 4.8 *M* urea (pH 2.3±0.05) and a voltage of 25 kV, both denatured β-Lg and *para*-κ-CN can be quantitatively and simultaneously determined in processed cheeses and other dairy products within 30 min.

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