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Determination of cows' milk in goats' milk and cheese by capillary electrophoresis of the whey protein fractions

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Abstract

The use of capillary zone electrophoresis to determine the adulteration of cows' milk in goats' milk products is described. The detection and quantification of cows' milk was based on the presence of the specific whey proteins: the relative calibration curve is reported. The peaks of interest were well resolved by using sodium borate at pH 9.2 as background electrolyte in methyl-silanized capillaries. The minimum amount detectable of cows' milk was 2% in milk mixtures and 4% in cheeses. Restrictions due to genetic variability and possible heat treatments, on only one of the two types of milk employed, are taken into account. Qualitative analysis of goat–ewe–cow and goat–ewe samples are also reported. © 1999 Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Milk and cheese products in general are among the most consumed foods in the world. The check of their quality requires accurate, sensitive and specific techniques that must also be quick and easy to carry out. These characteristics rarely all come together in one single analytical method and hence the continuous need to develop new methods.

The use of cows' milk in the production of cheeses that have been declared as only goats' milk is a profitable type of fraud that is simple to carry out and effectively found [1]. An effective testing technique should allow application not only to cheeses but also to milk samples, in order to safeguard cheese producers who purchase large amounts of milk from dairy farmers and to check goats' milk for direct consumption.

There have been many works on the subject in the literature, and they have used three main kinds of approach to the problem: immunological, via HPLC and electrophoresis. Enzyme-linked immunosorbent assay (ELISA) immunological methods [2,3] are the most sensitive and are not influenced by any heat treatment the sample undergoes, but routine use of the technique will only be possible when specific antibodies and quality-certified materials become commercially available.

HPLC methods use the reversed-phase technique to analyze whey proteins and, even with the limits of genetic variability and heat sensitivity, seem suitable for routine use. However, some of them reveal complex chromatograms [4], while others have long runtimes and do not detect the presence of goats' milk in triple mixtures of goat–ewe–cow milk and in double mixtures of goat–ewe milk [1]. Among the more traditional electrophoretic methods, mention must be made of a recent application of polyacrylamide gel electrophoresis (PAGE) and isoelec-

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tric focusing (IEF) in analyzing a typical cheese made of mixed goat–ewe–cow milk [5].

Capillary electrophoresis to analyze milk whey proteins has been successfully used for some years [6–11], and its speed and ease of use make it a highly competitive technique in the study of dairy products [12,13].

Capillary zone electrophoresis (CZE) has recently been used for analyzing only goat–ewe–cow milk mixtures, achieving a limit of detection of 8% of added cows' milk [14].

The method put forward in this article for the first time uses CZE for whey protein analysis of goat cheeses and achieves a limit of detection up to 2% of cows' milk present in mixed milk samples.

2. Experimental

All reagents used were of analytical-grade purity. The sodium hydroxide, hydrochloric acid, sodium tetraborate, sodium acetate, acetic acid, benzyl alcohol and methanol were supplied by Carlo Erba (Milan, Italy), while β-lactoglobulin A, β-lactoglobulin B and α-lactalbumin from bovine milk were all supplied by Sigma (St. Louis, MO, USA).

Analysis were carried out using a Spectraphoresis 1000 instrument from Thermo Quest (CA, USA). The capillaries used were of the methyl deactivated type: 50 μm I.D.×220 μm O.D. from SGE (Melbourne, Australia); total length was of 44 cm (37 to the detector) for cow–goat analysis and 68 cm (61 cm to the detector) for cow–goat–ewe and goat–ewe analysis.

2.1. Cheese and milk samples

The raw cow and goats' milk samples were obtained under our direct supervision from dairies in Italy. We made the cheeses ourselves following the typical procedure used in many Italian dairies and bearing in mind the experience gained in our previous works [12,13]: the right quantity of rennet was added to the milk sample at a temperature of 38°C. After 30 min of rest the sample was heated to reach a temperature of 45°C. The semisolid phase (curd) was then separated from the liquid phase (whey). Rennet in paste form from kid abomasum was used.

In this way, nine cheeses were produced: one cheese from goats' milk only, and eight cheeses produced with 4, 15, 20, 25, 40, 50, 60 and 80% of cows' milk. Seven days after production, cheese samples were prepared for CZE analysis. An appropriately diluted filtered rennet solution was injected under analysis conditions but no peaks were observed.

2.2. Sample preparation for CZE analysis

2.2.1. Milk samples

Ten ml of milk (25°C) were taken to pH 4.00 with 2 *M* HCl. After 20 min, the sample underwent centrifugation at 1800 *g* for 20 min (25°C); 2 ml of the supernatant (whey) were diluted with 1 ml of 0.2 *M* acetic buffer (adjusted at a pH of 4.00), and after 15 min a second centrifugation at 1800 *g* for 10 min (25°C) was carried out. The supernatant was filtered through a 0.45-μm filter (Millex-HV, Durapore membrane from Millipore, Milford, MA, USA) and then diluted with the same volume of Milli-Q-purified water. The sample was ready for injecting.

2.2.2. Cheese samples

Ten g of cheese were homogenized with 10 ml of Milli-Q-purified water. After 20 min, centrifugation of 20 min was carried out following the conditions described above; 2 ml of the supernatant were diluted with 1 ml of 0.2 *M* acetic buffer at a pH of 4.00. After 15 min a second centrifugation for 10 min and a filtration, using a Millipore 0.45-μm filter, were carried out. The sample was ready for injecting.

2.3. CZE operational conditions

Prior to use, the new capillary was conditioned with washes (0.1 *M* NaOH for 20 min, water for 5 min, 60 m*M* borate buffer for 5 min) alternating with runs in order to determine EOF (electroosmotic flow) measured with benzyl alcohol. The capillary was deemed ready for analysis as soon as the EOF became constant.

Samples were injected by using a pressure (hydrodynamic injection) of 10 340 Pa for 0.3 or 0.7 s. Others analysis conditions were the following: 4 or 6 kV (see figures and discussion for details), 50 m*M* borate buffer, pH 9.27, or 120 m*M* borate buffer, pH

9.20, as unique component of BGE (background electrolyte), 25°C, cathodic detection at λ of 200 nm. The capillaries required one wash per day before starting measurements (9 min with 0.1 *M* NaOH, 3 min with water, 3 min with BGE), and after every run the following washing was always performed: 0.1 *M* NaOH for 2 min, water for 2 min, methyl alcohol for 2 min, water for 2 min and BGE for 2 min.

3. Results and discussion

In the experimental conditions of Fig. 1 the cow α-lactalbumin shows the same migration time (t_M) as one of the two goat whey proteins. Therefore the electropherogram of a goat–cow milk mixture (Fig. 2) reveals four peaks, the first of which relate to a goat protein (G1) and the last relating to the cow protein β-lactoglobulin A (β-LGA). These two substances can thus be usefully employed as markers for the presence of the corresponding milk sources. Goat peak G1 was considered relative to goat α-lactalbumin (α-LA) due to its quantity, which was lower than that of the substance relating to peak G2: normally goats' milk has more β-LG than α-LA, and so α-LA could be the less intense peak. Where necessary, pure goats' milk was used as a standard.

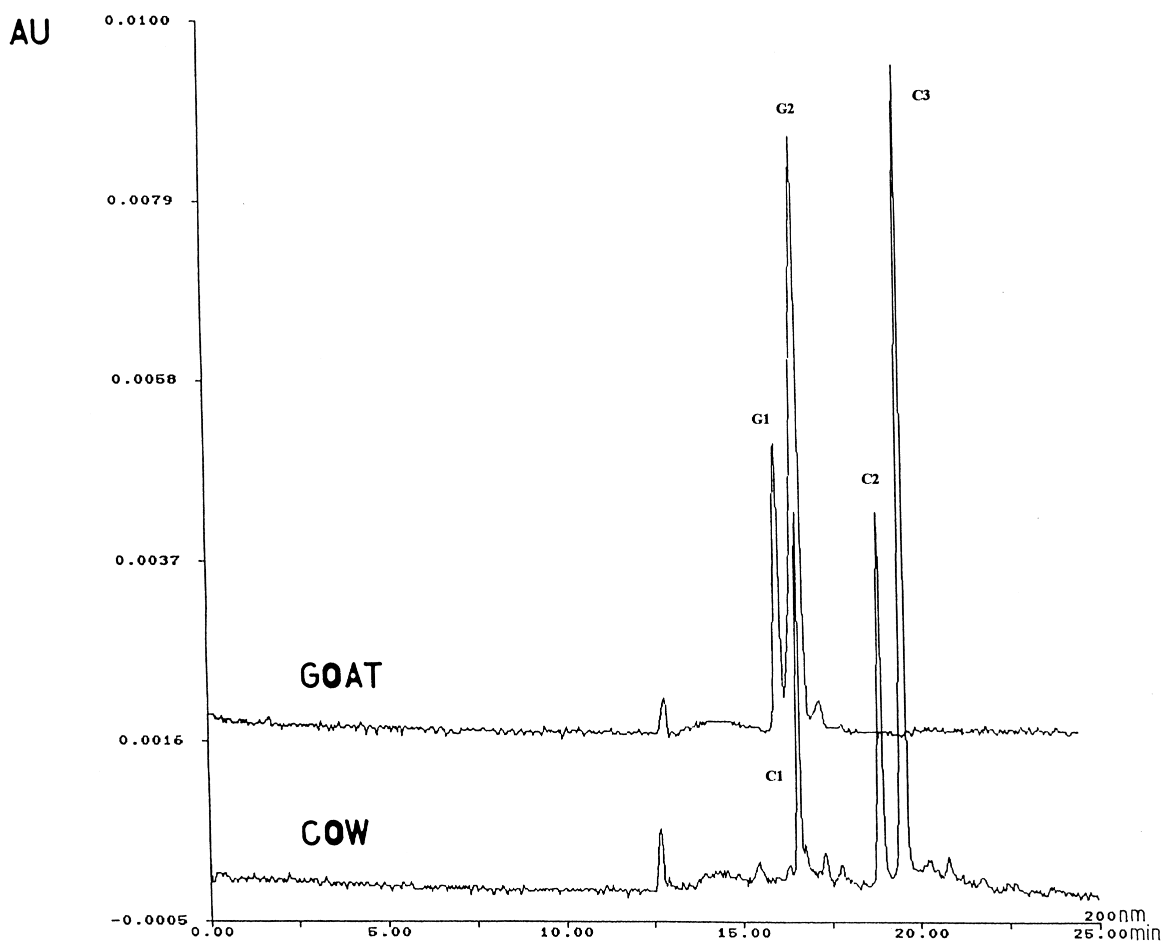

Fig. 1. Electropherograms of whey from goats' milk and cows' milk. Experimental conditions: methyl-silanized capillary (total length, 44 cm×50 μm I.D.), 50 m*M* borate buffer, 4 kV, 25°C, cathodic detection (λ=200 nm). Peaks: G1, goat α-lactalbumin; G2, goat β-lactoglobulin; C1, cow α-lactalbumin; C2, cow β-lactoglobulin B; and C3, cow β-lactoglobulin A.

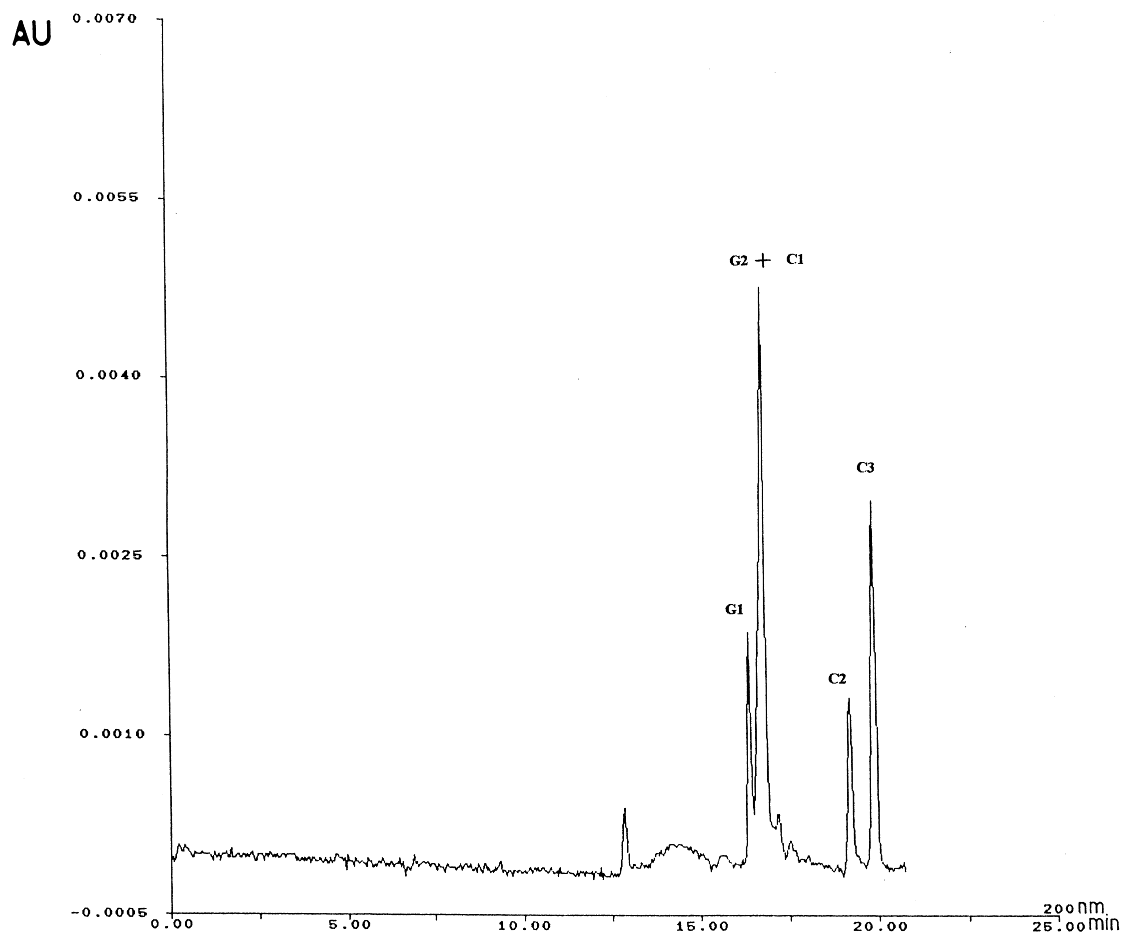

Fig. 2. Electropherogram of whey from a goat–cow milk mixture sample (60% of goats' milk, 40% of cows' milk). Conditions and peaks as in Fig. 1.

The ratio

$$R = \frac{\text{corrected area cow } \beta - \text{LGA}}{\text{corrected area goat } \alpha - \text{LA}}$$

where

$$\text{corrected area} = \frac{\text{peak area}}{\text{migration time}}$$

was used as a reference parameter in the quantitative analysis: as the cows' milk component present in the mixture approached zero, the value of R also approached zero, while the latter value approached infinity as the cows' milk component approached 100. Of the two cows' whey proteins, both well resolved, preference was given to β-LGA since it is an exclusive characteristic of cows' milk (whereas β-LGB is also found in buffalo milk [12]).

Moreover, β-LGA is constantly seen to be more intense than β-LGB, thus allowing a lower limit of detection. A calibration curve was experimentally constructed in order to relate the value of R to sample composition. Twenty-four calibration samples were used (15 milk mixtures and nine mixed cheeses). Three consecutive determinations of the R value were carried out on each sample; the average of this value, reported in the graph as a function of the cows' milk present in the sample, yielded the calibration curve of Fig. 3. The excellent linearity of the method's response in the range 0–20% must be noted, where the calibration curve can be approxi-

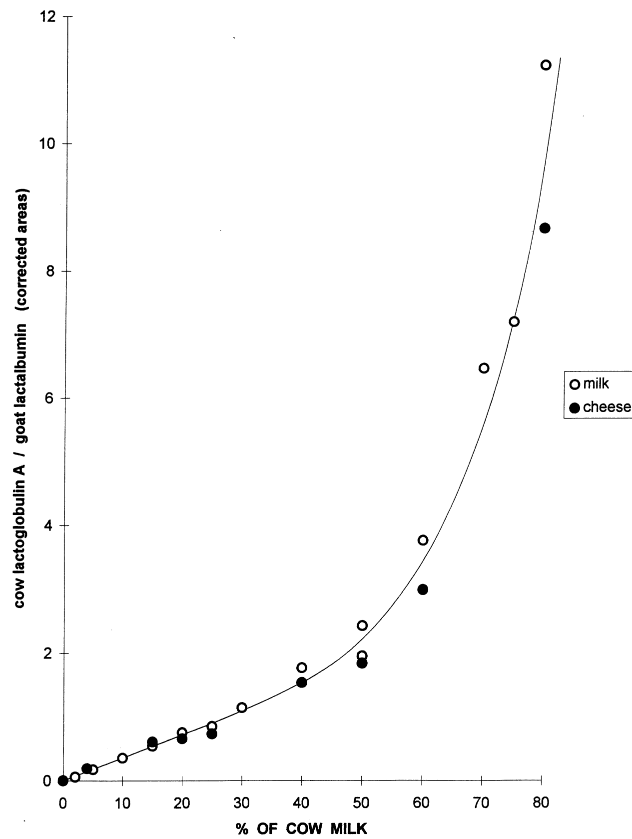

Fig. 3. Calibration curve for determining the amount of cows' milk in goats' milk products.

Table 1
Repeatability of migration times and peak areas of a milk mixture sample

Injection	Goat α-lactalbumin			Cow β-lactoglobulin A			R^a	Cows' milk (%)
	t_M (min)	Area (area counts)	Area/t_M^b (area counts/min)	t_M (min)	Area (area counts)	Area/t_M^b (area counts/min)		
1	16.24	55 684	3429	19.74	49 043	2484	0.72	19.89
2	16.26	52 452	3226	19.75	47 919	2426	0.75	20.72
3	16.44	51 892	3156	20.01	48 095	2404	0.76	21.00
4	16.22	54 342	3350	19.67	45 782	2328	0.69	19.05
5	16.53	55 983	3387	20.13	49 914	2480	0.73	20.16
Mean	16.34	54 071	3310	19.86	48 151	2424	0.73	20.16
S.D.	0.14	1851	115	0.20	1574	64	0.03	0.76
R.S.D. (%)	0.85	3.42	3.46	1.00	3.21	2.64	3.75	3.78

Conditions as in Fig. 1.
[a]Ratio of the corrected peak areas.
[b]Corrected peak area.

mated to a straight line with $r^2 = 0.98$ and equation $y = 0.0359x + 0.0061$ (linear regression).

The data points relative to the milk samples and the ones relative to the cheese samples practically lie on the same calibration curve, confirming what was seen in our previous works: the process leading from the milk mixture to the finished cheese does not alter the whey protein ratio and thus the value of R [12,13]. Therefore it is possible to use the same calibration for both products. The minimum quantity of cows' milk component detectable is 2% in milk mixtures and 4% in mixed cheeses, considering a signal-to-noise ratio of 3.

Repeatability and precision were assessed by carrying out five consecutive injections of the same sample (Table 1).

The mean error (accuracy) of the data point is very low in the range 0–20% (±1) and only slightly higher in the range >20% (±3), but two particular cases must be taken into account: if the protein levels of the pure milks that make up the sample to be analyzed are different from the protein levels of the pure milks used for the calibration curve, the error made in determining the cows' milk percentage may be considerable (protein levels may vary when moving from a goat or cow breed to some other

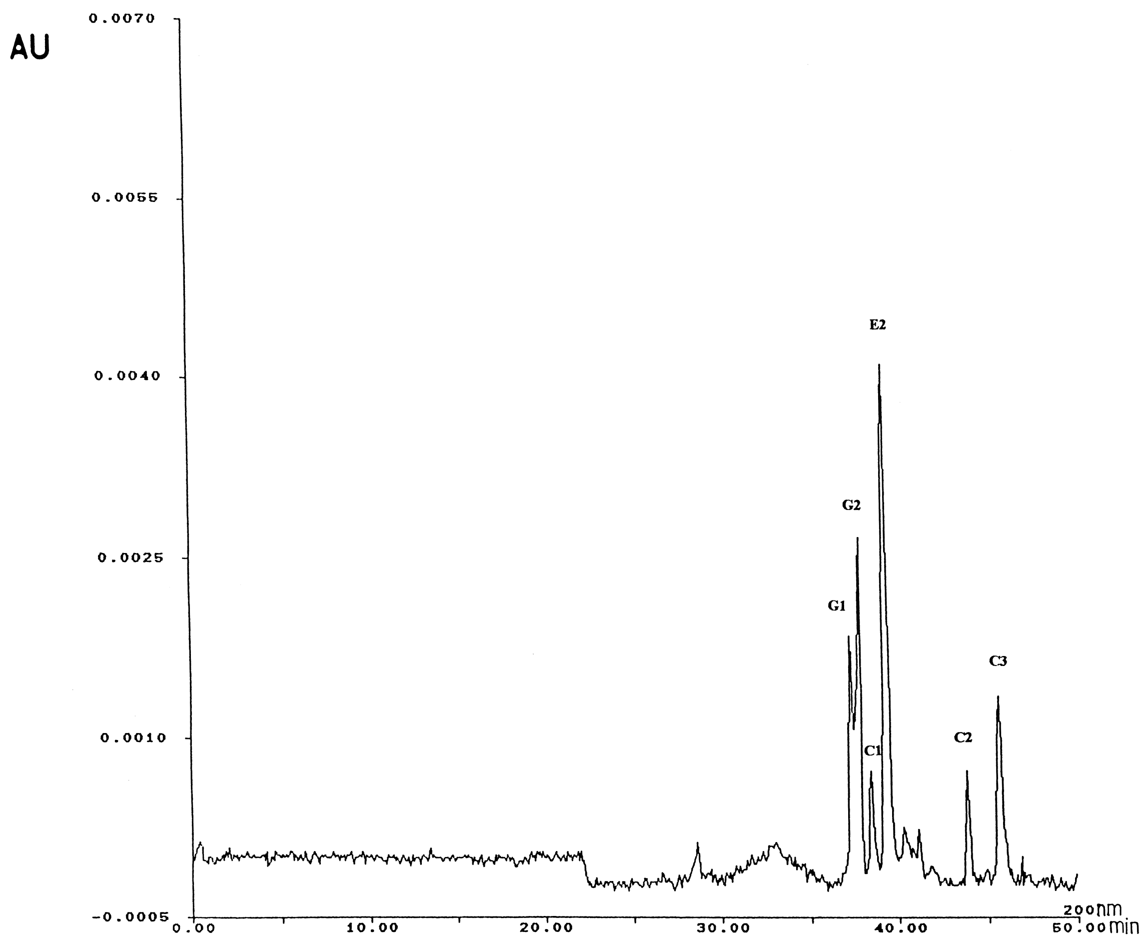

Fig. 4. Electropherogram of whey from a ternary milk mixture (40% of goats' milk, 30% of ewes' milk, 30% of cows' milk). Experimental conditions: methyl-silanized capillary (total length, 68 cm×50 μm I.D.), 120 mM borate buffer, 6 kV, 25°C, cathodic detection ($\lambda = 200$ nm). Peaks: G1, goat α-lactalbumin; E1, ewe α-lactalbumin; C1, cow α-lactalbumin; G2, goat β-lactoglobulin; E2, ewe β-lactoglobulin; C2, cow β-lactoglobulin B; and C3, cow β-lactoglobulin A.

breed—genetic variability—while there is less variation generally when going from one individual to another of the same breed); moreover, a heat treatment on the cows' milk used in producing the sample can lead to underestimating its quantity. This last issue, however, does not include pasteurization of milk, which is always carried out when producing cheese. Recent work demonstrated that pasteurization at 74°C for 15 or 30 s, normally employed by cheese producers, does not influence the determination of cows' milk percentage fraudulently added to products declared to be made 'of pure goats' milk' [5].

The method was applied to five commercial samples of cheese that showed the composition declared by the producers. All the samples were fresh cheese varieties: i.e., in the form in which goats' milk cheese is mainly consumed, and thus peptides from the ripening process [13] were hardly ever seen. For this reason, the electropherograms of goats' milk cheeses were very similar to that of the original milk they were derived from.

3.1. Other mixtures

Considering the widespread consumption of products declared to be of 'mixed goat–sheep' origin and the presence of declared triple mixtures (goat–cow–ewe milk) on the market, we wished to study the possibility of analyzing these samples with the same type of methyl-silanized capillary and using borate such as BGE. As shown in Fig. 4, the resolution of the maximum number of bands in the three-milk mixtures (goat–ewe–cow) is obtained by increasing the column length (the number of theoretical plates increases) and by increasing the voltage (on which the resolution directly depends). BGE concentration was increased in order to maintain peak efficiency and prevent protein adsorption on the capillary wall; these phenomena can become quite considerable when the bands stay a long time in the column, as in this case. Under these conditions, the appearance of the electropherogram of a three-milk mixture is very good, but (unlike that which was seen for all the other peaks) there is a loss of resolution between the two goats' milk peaks, G1 and G2. This fact, together with the overlap between peaks E1 (ewes' milk) and G1 (goat), does not allow accurate quantitative analysis on triple mixtures and on double mixtures of goat–ewe milk. Qualitative analysis, for identification of the kinds of milk present in the sample is, instead, always possible to carry out.

4. Conclusions

Considering one of our previous works on ewe–cow milk products [13] we may conclude by saying that the use of a borate buffer and methyl-silanized capillaries allows an analysis—via CZE—of products deriving from all possible goat–ewe–cow milk combinations; only qualitative analysis is possible for three-milk mixtures and goat–ewe milk mixtures products.

These analysis methods through CZE are also the quickest currently available and, thanks to their characteristics, are expected to see widespread routine use for their ease and reliability.

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