COMMISSION REGULATION (EEC) No 690/92

of 19 March 1992

establishing a reference method for the detection of cows' milk casein in cheeses made from ewes' milk

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community,

Having regard to the Treaty of Accession of Spain and Portugal and in particular Article 67 (1) and (2), Article 98, Article 234 (2) and Article 310 thereof,

Having regard to Council Regulation (EEC) No 1677/85 of 11 June 1985 on monetary compensatory amounts in the agricultural sector [1], as last amended by Regulation (EEC) No 2205/90 [2], and in particular Article 1 (1) thereof,

Having regard to Council Regulation (EEC) No 804/68 of 27 June 1968 on the common organization of the market in milk and milk products [3], as last amended by Commission Regulation (EEC) No 374/92 [4], and in particular Article 9 (3), Article 14 (7) and Article 17 (4) thereof,

Having regard to Council Regulation (EEC) No 876/68 of 28 June 1968 on the general rules for granting export refunds in the milk and milk products sector and for the fixation of their amounts [5], as last amended by Regulation (EEC) No 1344/86 [6], and in particular Article 6 (3) thereof,

Whereas cheeses made exclusively from ewes' milk are subject to certain specific rules under the terms of Community regulations in the agricultural sector; whereas prior to the application of these rules steps should be taken to check that the product concerned does not contain cows' milk;

Whereas aid for private storage of ewes' milk cheeses can be granted under Council Regulation (EEC) No 508/71 of 8 March 1971 laying down general rules on private storage aid for long-keeping cheeses [7]; whereas a special refund for these same products can be decided under the terms of Regulation (EEC) No 876/86; whereas no monetary compensatory amounts are imposed for the trade in ewes' milk-based products under the terms of Regulation (EEC) No 1677/85; whereas the application of accessionary compensatory amounts is excluded trade in ewes' milk cheeses to and from Spain by Council Regulation (EEC) No 466/86 of 25 February 1986 determining the general rules for the application of accessionary compensatory amounts in the milk and milk products sector with respect to the accession of Spain [8], and by Council Regulation (EEC) No 3640/90 of 11 December 1990, determining the general rules for the application of accessionary compensatory amounts in the milk and milk products sector with respect to the accession of Portugal [9];

Whereas, as the Commission has indeed established the abovementioned provisions concerning cheeses made from ewes' milk, it is necessary to verify by appropriate control that no cows' milk has been incorporated into the products concerned; whereas it appears necessary at control level to establish a Community reference method for the detection of cows' milk without prejudice to the use of routine methods, if they comply with certain criteria;

Whereas the Management Committee for Milk and Milk Products has not delivered an opinion within the time limits set by its chairman,

HAS ADOPTED THIS REGULATION:

Article 1

The reference method of analysis figuring in the Annex shall be applied to ensure that cheese which must be exclusively made from ewes' milk does not in fact contain cows' milk casein.

Cows' milk casein shall be held to be present, if the apparent cows' milk casein content of the sample to be analyzed is equal to or higher than the content of the reference sample described in the Annex.

Article 2

Routine methods for the detection of cows' milk casein in cheeses from ewes' milk may be used under the following conditions:

— the detection limit must be 0,5 % or lower,

— false-positive results must not occur. If this cannot be excluded, any sample giving a positive result has to be analyzed using the reference method,

[1] OJ No L 164, 24. 6. 1985, p. 6.
[2] OJ No L 201, 31. 7. 1990, p. 9.
[3] OJ No L 148, 28. 6. 1968, p. 13.
[4] OJ No L 41, 18. 2. 1992, p. 9.
[5] OJ No L 155, 3. 7. 1968, p. 1.
[6] OJ No L 119, 8. 5. 1986, p. 36.
[7] OJ No L 58, 11. 3. 1971, p. 1.
[8] OJ No L 53, 1. 3. 1986, p. 23.
[9] OJ No L 362, 27. 12. 1990, p. 3.

— cows' milk casein must be detectable with the required sensitivity even after long ripening periods, as may occur under usual commercial conditions. If this requirement is not fulfilled for a certain type of ewes' milk cheese, that cheese must be analyzed using the reference method.

Article 3

This Regulation shall enter into force on the third day following its publication in the *Official Journal of the European Communities.*

It shall apply from 16 September 1992.

This Regulation shall be binding in its entirety and directly applicable in all Member States.

Done at Brussels, 19 March 1992.

For the Commission

Ray MAC SHARRY

Member of the Commission

ANNEX

REFERENCE METHOD FOR THE DETECTION OF COWS' MILK CASEIN IN CHEESES MADE FROM EWES' MILK

1. **Scope**

Detection of cows' milk casein in cheeses made from ewes' milk by isoelectric focusing of y-caseins after plasminolysis.

2. **Field of application**

The method is suitable for sensitive and specific detection of cows' milk casein in fresh and ripened cheeses made from ewes' milk.

3. **Principle of the method**

3.1. Isolation of caseins from cheese.

3.2. Dissolving of the isolated caseins and submitting to plasmin (EC.3.4.21.7) cleavage.

3.3. Isoelectric focusing of plasmin-treated caseins in the presence of urea and straining of proteins with Coomassie Brilliant Blue G-250.

3.4. Evaluation of stained γ_2-casein patterns (evidence of cows' milk) by comparison of the pattern obtained from the sample with those obtained in the same gel from standards containing 0 % and 1 % cows' milk.

4. Reagents

Unless otherwise indicated, analytical grade chemicals must be used. Water must be double-distilled or of equivalent purity.

Note: The following details apply to laboratory prepared polyacrylamide gels containing urea, of dimensions 265 × 125 × 0,25 mm. Where other sizes and types of gel are used, the separation conditions may have to be adjusted.

Isoelectric focusing

4.1. Reagents for production of the urea containing polyacrylamide gels

4.1.1. Stock gel solution

Dissolve

4,85 g acrylamide

0,15 g N,N'-methylene-bis-acrylamide (BIS)

48,05 g urea

12,22 g glycerol (87 % w/w)

in water and make up to 100 ml and store in a brown glass bottle in the refrigerator.

Note: A commercially available preblended acrylamide/BIS solution can be used in preference to the quoted fixed weights of the neurotoxic acrylamides. Where such a solution contains 30 % w/v acrylamide and 0,8 w/v BIS, a volume of 16,2 ml must be used for the formulation instead of the fixed weights. The shelf life of the stock solution is a maximum of 10 days ; if its conductivity is more than 5µS, de-ionize by stirring with 2 g Amberlite MB-3 for 30 min, then filter through a 0,45 µm membrane.

4.1.2. Gel solution

Prepare a gel solution by mixing additives and ampholytes with the stock gel solution (see 4.1.1).

9,0 ml stock solution

24 mg ß-alanine

500 µl ampholyte pH 3,5-9,5 (1)

500 µl ampholyte pH 6-7 (1)

Mix the gel solution and de-gas for two to three minutes in an ultrasonic bath or in vacuo.

Note: Prepare the gel solution immediately prior to pouring it (see 6.2).

(1) The products Ampholine pH 3,5-9,5 (Pharmacia-LKB) and Servalyte pH 6-7 (Serva) have proved particularly suitable for obtaining the required separation of y-caseins.

4.1.3. Catalyst solutions

40 % w/v ammonium persulphate (PER):

Dissolve 800 mg PER in water and make up to 2 ml.
N,N,N'N'-tetramethylethylene diamine (TEMED).

Note: Always use freshly prepared PER solution.

4.2. Contact fluid

Kerosene or liquid paraffin.

4.3. Anode solution

Dissolve 5,77 g phosphoric acid (85 % w/w) in water and dilute to 100 ml.

4.4. Cathode solution

Dissolve 2,00 g sodium hydroxide in water and dilute to 100 ml with water.

Sample Preparation

4.5. Protein isolation reagents

Dichloromethane.
Dilute acetic acid (25,0 ml of glacial acetic acid made up to 100 ml with water).
Acetone.

4.6. Protein dissolving buffer

Dissolve
5,75 g glycerol (87 % w/w)
24,03 g urea
250 mg dithiothreitol
in water and make up to 50 ml

Note: Store in a refrigerator, maximum shelf-life 1 week.

4.7. Reagents for Plasmin cleavage of casein

4.7.1. Ammonium carbonate buffer

Titrate a 0,2 mol/l ammonium hydrogencarbonate solution (1,58 g/100 ml water) with a 0,2 mol/l ammonium carbonate solution (1,92 g/100 ml water) to pH 8.

4.7.2. Bovine plasmin (E.C. 3.4.21,7), activity at least 5 U/ml.

4.7.3. Enzyme inhibition solution

Dissolve 2,624 g ε-aminocaproic acid (6-amino-n-hexanoic acid) in 100 ml of 40 % (v/v) ethanol.

4.8. Preparation of the renneted ewes' skimmed milk standards containing 0 % and 1 % of cows' milk

Skimmed milk is prepared by centrifuging of ovine or bovine raw bulk milk at 37° C at 2 500 g for 20 min. After cooling the tube and contents rapidly to 6-8° C, the upper fat layer is removed completely. For the preparation of the 1 % standard add 5,00 ml of bovine skimmed milk to 495 ml of ovine skimmed milk in a one-litre beaker, adjust the pH to 6,4 by the addition of dilute lactic acid (10 % w/v). Adjust the temperature to 35° C and add 100 µl of calf rennet (rennet activity 1 :10 000, c. 3 000 U/ml), stir for one minute and then leave the beaker covered with an aluminium foil at 35° C for one hour to allow formation of the curd. After the curd has formed, the whole renneted milk is freeze-dried without prior homogenization or draining of the whey. After freeze-drying it is finely ground to produce a homogeneous powder. For the preparation of the 0 % standard, carry out the same procedure using 300 ml of pure ovine skimmed milk.

Note: It is advisable to check the purity of the ovine milk by isoelectric focusing of the plasmin-treated caseins before preparation of the standards.

Reagents for protein staining

4.9. Fixative

Dissolve 150 g trichloroacetic acid in water and make up to 1 000 ml.

4.10. Destaining solution

Dilute 500 ml methanol and 200 ml glacial acetic acid to 2 000 ml with distilled water.

Note: Prepare the destaining solution fresh every day; it can be prepared by mixing equal volumes of stock solutions of 50 % (v/v) methanol and 20 % (v/v) glacial acetic acid.

4.11. Staining solutions

4.11.1. Staining solution (stock solution 1)

Dissolve 3,0 g Coomassie Brilliant Blue G 250 (C.I. 42655) in 1 000 ml 90 % (v/v) methanol using a magnetic stirrer (± 45 minutes), filter through two medium-speed folded filters.

4.11.2. Staining solution (stock solution 2)

Dissolve 5,0 g copper sulphate pentahydrate in 1 000 ml 20 % (v/v) acetic acid.

4.11.3. Staining solution (working solution)

Mix together 125 ml of each of the stock solutions (4.11.1, 4.11.2) immediately prior to staining.

Note: The staining solution should not be re-used other than on the day of preparation.

5. **Equipment**

5.1. Glass plates (265 × 125 × 4 mm); rubber roller (width 15 cm); levelling table.

5.2. Gel carrier sheet (265 × 125 mm).

5.3. Covering sheet (280 × 125 mm). Stick one strip of adhesive tape (280 × 6 × 0.25 mm) to each long edge (see Fig. 1).

5.4. Electrofocusing chamber with cooling plate (e.g. 265 × 125 mm) with suitable voltage source (≥ 2,5 kV) or automatic electrophoresis device.

5.5. Circulation cryostat, thermostatically controlled at 12 ± 0.5° C.

5.6. Centrifuge, adjustable to 3 000 g.

5.7. Electrode strips (≥ 265 mm long).

5.8. Plastic dropping bottles for the anode and cathode solutions.

5.9. Sample applicators (10 × 5 mm, viscose or low protein-absorption filter paper).

5.10. Stainless steel scissors, scalpels and tweezers.

5.11. Stainless steel or glass staining and destaining dishes (e.g. 265 × 150 mm instrument trays).

5.12. Adjustable rod homogenizer (10 mm shaft diameter), rpm range 8 000-20 000.

5.13. Magnetic stirrer.

5.14. Ultrasonic bath.

5.15. Film welder.

5.16. 5-25 µl micropipettes.

5.17. Vacuum concentrator or freeze-dryer.

5.18. Thermostatically controlled water bath adjustable to 35 and 40 ± 1° C with shaker.

5.19. Densitometer equipment reading at λ = 634 nm.

6. **Procedure**

6.1. Sample preparation

6.1.1. Isolation of caseins

Weigh the amount equivalent to 5 g dry mass of cheese or standards — for white mould cheese where possible use the unripe centre — into a 100 ml centrifuge tube, add 60 ml distilled water and homogenize with a rod homogenizer (8 000-10 000 rpm). Adjust to pH 4,6 with dil. acetic acid (4.5) and centrifuge (five minutes, 3 000 g). Decant the fat and whey, homogenize the residue at 20 000 rpm in 40 ml distilled water adjusted to pH 4-5 with dil. acetic acid (4.5), add 20 ml dichloromethane (4.5), homogenize again and centrifuge (five minutes, 3 000 g). Remove the casein layer that lies between the aqueous and organic phases (see figure 2) with a spatula and decant off both phases. Re-homogenize the casein in 40 ml distilled water (see above) and 20 ml dichloromethane and centrifuge. Repeat this procedure until both extraction phases are colourless (two to three times). Homogenize the protein residue with 50 ml dry acetone (4.5) and filter through a medium-speed folded filter paper. Wash the residue on the filter with two separate 25 ml portions of acetone each time and allow to dry in the air or a stream of nitrogen, then pulverize finely in a mortar.

Note: Dry protein isolates can be kept indefinitely at room temperature. For rapid protein isolation homogenize the amount equivalent to 5 g dry mass of cheese twice with each 100 ml acetone (4.5) at 20 000 rpm, leave to stand for five minutes and then filter off through a folded filter. Dry the protein residue with acetone as described above to finally obtain the acetone-dried powder. The rapid method is not applicable to Roquefort-type cheese.

6.1.2. Plasmin cleavage of ß-caseins to intensify γ-caseins

Suspend 25 mg isolated casein or 50 mg of the freeze-dried standards (4.8) or the acetone-dried powder from rapid protein isolation in 0,5 ml ammonium carbonate buffer (4.7.1) and homogenize for 20 min. by e.g. using ultrasonic treatment. Heat to 40° C and add 100 µl plasmin (4.7.2), mix and incubate for one hour at 40° C with continuous shaking. To inhibit the enzyme add 20 µl ε-aminocaproic acid solution (4.7.3), then add 200 mg of solid urea and 2 mg of dithiothreitol.

Note: To obtain more symmetry in the focused casein bands it is advisable to freeze-dry the solution after adding the ε-aminocaproic acid and then dissolving the residues in 0,5 ml urea buffer (4.6).

6.2. Preparation of the urea containing acrylamide gels

With the aid of a few drops of water roll the gel carrier sheet (5.2) onto a glass plate (5.1), removing any extraneous water with paper towel or tissue. Roll the cover sheet (5.3) with spacers (0,25 mm) onto another glass plate in the same way. Lay the plate horizontally on a levelling table.

Add 10 µl each of the catalyst solutions Temed and PER (4.1.3) to the prepared and de-aerated gel solution (4.1.2), mix briefly and pour out evenly onto the centre of the cover sheet. Place one edge of the gel carrier plate (sheet side down) on the cover sheet plate and lower it slowly so that a gel film forms between the sheets and spreads out regularly and free of bubbles (Fig. 3). Carefully lower the gel carrier plate completely using a thin spatula and place three more glass plates on top of it to act as weights. After polymerization is complete (about 60 minutes) remove the gel polymerized onto the gel carrier sheet along with the cover sheet by tipping the glass plates. Clean the reverse of the carrier sheet carefully to remove gel residues and urea. Weld the 'gel sandwich' into a film tube and store in a refrigerator (maximum six weeks).

Note: The cover sheet with the spacers can be re-used. The polacrylamide gel can be cut to smaller sizes, recommended when there are few samples or if an automatic electrophoresis device is used (two gels, size 4,5 × 5 cm).

6.3. Isoelectronic focusing

Set the cooling thermostat to 12° C. Wipe off the reverse of the gel carrier sheet with kerosene, then drip a few drops of kerosene (4.2) onto the centre of the cooling block. Then roll the gel sandwich, carrier side down, onto it, taking care to avoid bubbles. Wipe off any excess kerosene and remove the cover sheet. Soak the electrode strips with the electrode solutions (4.3, 4.4), cut to gel length and place in the positions provided (distance of electrodes 9,5 cm).

Conditions for isoelectric focusing:

6.3.1. Gel size 265 × 125 × 0,25 mm

Step	Time (minutes)	Voltage (V)	Current (mA)	Power (W)	Volt hours (Vh)
1. Prefocusing	30	maximum 2 500	maximum 15	constant 4	± 300
2. Sample focusing (*)	60	maximum 2 500	maximum 15	constant 4	± 1 000
3. Final focusing	60	maximum 2 500	maximum 5	maximum 20	± 3 000
	40	maximum 2 500	maximum 6	maximum 20	± 3 000
	30	maximum 2 500	maximum 7	maximum 25	± 2 500

(*) Sample application: After prefocusing (step 1), pipette 18 µl of each of the sample solutions onto the sample applications (10 × 5 mm), place them on the gel at 1 mm intervals from each other and 5 mm longitudinally from the anode and press lightly. Carry out focusing using the above conditions, carefully removing the sample applicators after the 60 minutes of sample focusing.

Note: If thickness or width of the gels are changed, the values for current and power have to be suitably adjusted (e.g. double the values for electric current and power if a 265 × 125 × 0.5 mm gel is used).

6.3.2. Example of a voltage programme for an automatic electrophoresis device (2 gels of 5.0 × 4.5 cm), electrodes without strips applied directly to the gel.

Step	Voltage	Current	Power	Temperature	Volt hours
1. Prefocusing	1 000 V	10,0 mA	3,5 W	8° C	85 Vh
2. Sample focusing	250 V	5,0 mA	2,5 W	8° C	30 Vh
3. Focusing	1 200 V	10,0 mA	3,5 W	8° C	80 Vh
4. Focusing	1 500 V	5,0 mA	7,0 W	8° C	570 Vh

Place sample applicator in step 2 at 0000 Vh

Remove sample applicator in step 2 at 0030 Vh

6.4. Protein staining

6.4.1. Protein fixation

Remove the electrode strips immediately after turning off the power and put the get immediately into a staining/destaining dish filled with 200 ml fixative (4.9); leave for 15 minutes, shaking occasionally.

6.4.2. Washing and stainng the gel plate

Thoroughly drain off the fixative and wash the gel plate twice for 30 seconds each time with 100 ml destaining solution (4.10). Pour off the destaining solution and fill the dish with 250 ml staining solution (4.11.3); allow to stain for 45 minutes with gentle shaking.

6.4.3. Destaining the gel plate

Pour off the staining solution, wash the gel plate twice with 100 ml destaining solution each time, then shake for at least 2 × 15 minutes with 200 ml destaining solution until the background is clear and uncoloured. Then rinse the gel plate with distilled water (2 × 2 minutes) and dry in the air (2-3 hours) or with a haridryer (10-15 minutes).

Note: Carry out fixing, washing, staining and destaining at 20° C. Do not use elevated temperatures.

7. Evaluation

Evaluation is performed by comparing the protein patterns of the sample with reference samples on the same gel. Detection of cows' milk in ewes' milk or the products made from them is done via the γ_2 and γ_3-caseins intensified by plasmin treatment (see 6.1.2), whose isoelectric points range between pH 6,5 and pH 7,5; see 4 and 5). The detection limit is below 0,5 %. For visual evaluation of the amount of bovine milk it is advisable to adjust the concentrations of samples and standards to obtain the same level of intensity of the ovine γ_2-caseins (see 'γ_2 S' in 4 and 5). After which the amount of bovine milk (less than, equal to or greater than 1 %) in the unknown sample can judged directly by comparing the intensity of the bovine γ_2-caseins (see 'γ_2 C' in 4 and 5). If available, apply densitometry (5.19) for the determination of the peak area ratio of bovine to ovine γ_2-caseins (see 5). Compare this value to the ratio of γ_2-caseins in the 1 % standard analyzed on the same gel.

Note: The method is operating satisfactory, if there is a clear positive signal for bovine γ_2-casein in the 1 % standard but not in the 0 % standard. If not, optimize the procedure following the details of the method precisely.

8. References

Addeo F., Moio L., Chianese L., Stingo C., Resmini P., Berner I., Krause I., Di Luccia A., Bocca A.: *Use of plasmin to increase the sensitivity of the detection of bovine milk in ovine cheese by gel isoelectric focusing of γ_2-caseins.* Milchwissenschaft 45, 708-711 (1990).

Krause I., Berner I., Klostermeyer H.: *Sensitive detection of cow milk in ewe and goat milk and cheese by carrier ampholyte — and carrier ampholyte/immobilized pH gradient — isoelectric focusing of γ_2-caseins using plasmin as signal amplifier.* In: Electrophoresis-Forum '89 (B.J. Radola, ed.) pp 389-393, Bode-Verlag, Munich (1989).

Krause I., Belitz H.-D., Kaiser K.-P.: *Nachweis von Kuhmilch in Schaf- und Ziegenmilch bzw. -käse durch isoelektrische Fokussierung in harnstoffhaligen Polyacrylamidgelen.* Z. Lebensm. Unters. Forsch. 174, 195-199 (1982).

Krause I., Berner I., Klostermeyer H.: Z. Lebensm. Unters. Forsch. (in preparation).

Radola B.J.: *Ultrathin-layer isoelectric focusing in 50-100 µm polyacrylamide gels on silanized glass plates or polyester films.* Electrophoresis 1, 43-56 (1980).

Figure 1 : Schematic drawing of the covering sheet

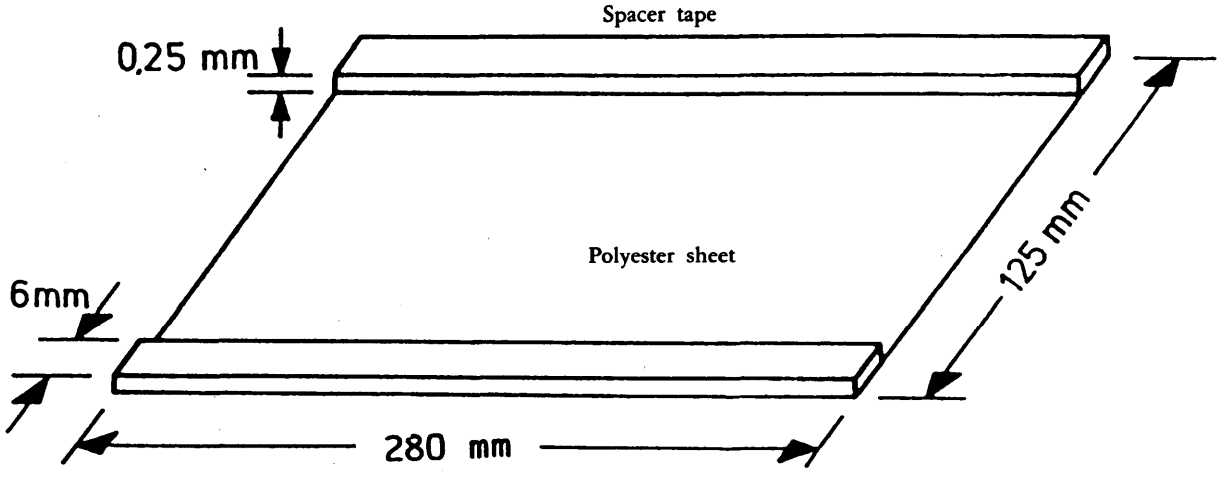


Figure 2 : Casein layer floating between aqueous and organic phases after centrifugation

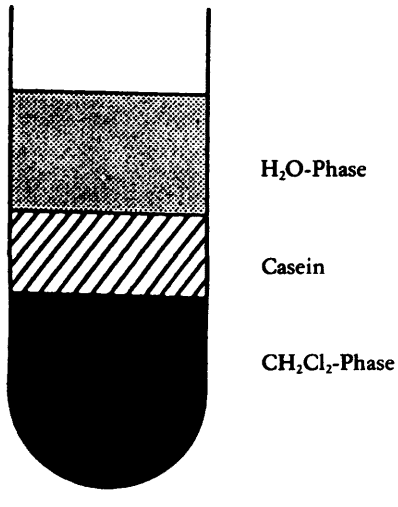


Figure 3 : Flapping technique for casting of ultrathin polyacrylamide gels.
a = spacer tape (0,25 mm) ; b = covering sheet (5.3) ; c, e = glass plates (5.1) ; d = gel solution (4.1.2) ; f = gel carrier sheet (5.2)

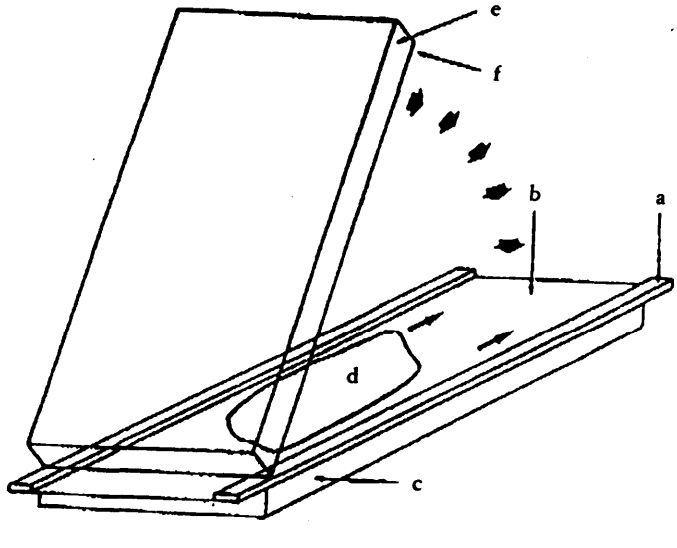


Figure 4 : Isoelectric focusing of plasmin-treated caseins from Pecorino-type cheese with different amounts of cows' milk. % CM = percentage of cow's milk, C = cow, S = sheep

Pecorino-type cheese containing :

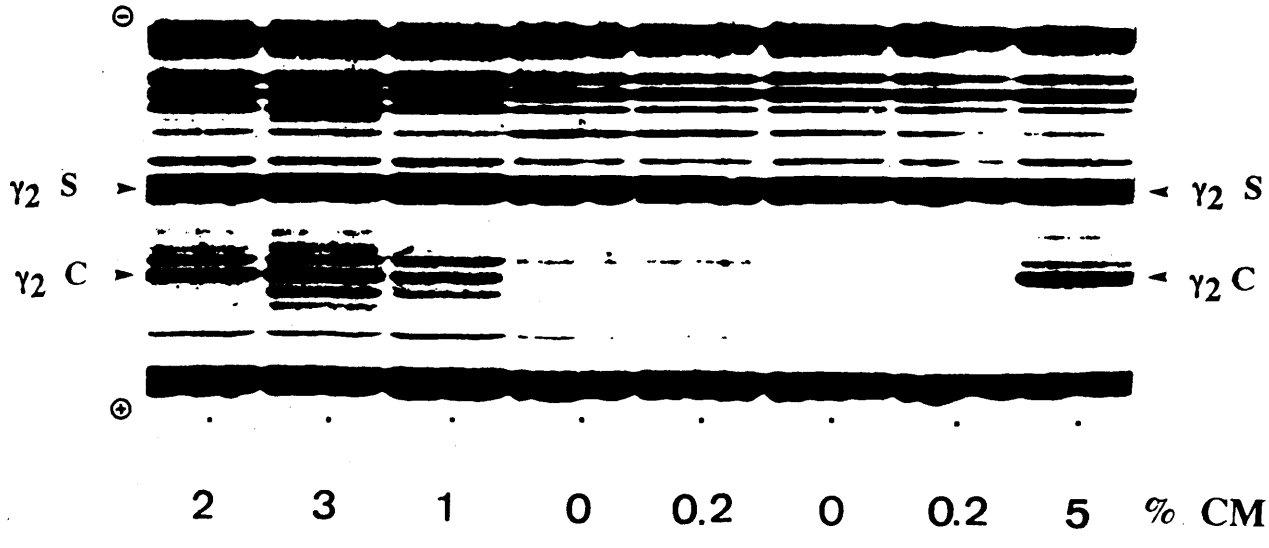


Figure 5: Superposition of densitograms of Pecorino-type cheese samples containing 0, 1, 2, 3 and 7 % of cows' milk after isolelectric focusing. Upper half of the IEF gel was scanned at λ = 634 nm. STD = standards containing 0 and 1 % of cows' milk

