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Fast detection of bovine milk in Roquefort cheese with PhastSystem® by gel isoelectric focusing and immunoblotting

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Summary — A fast procedure for the detection of bovine milk in Roquefort cheese is described. It is based on the separation by rapid isoelectric focusing on PhastSystem® apparatus of γ_2-caseins from the milk of the 2 species. The presence of bovine milk is also confirmed by the detection in the electrophoretic pattern of a β-casein derived peptide from bovine milk, during cheese ripening, identified by immunoblotting. By using this procedure, levels of bovine milk as low as 5% were easily detected in Roquefort cheese ripened for a period varying from 10 days to 5 months.

isoelectric focusing / PhastSystem® / Roquefort cheese / immunoblotting

Résumé — Détection rapide de lait de vache dans le fromage Roquefort par isoélectrofocalisation rapide et immunoblotting avec PhastSystem®. *Cet article décrit une procédure rapide de détection du lait de vache utilisé dans la fabrication du fromage Roquefort. Cette procédure est basée sur la séparation de la caséine γ_2 bovine et ovine par isoélectrofocalisation rapide avec un appareil du type PhastSystem®. La présence du lait de vache est confirmée par la présence dans le profil d'électrophorèse d'une bande supplémentaire identifiée par la méthode d'immunoblotting. Cette bande est dérivée de la caséine β bovine au cours de la maturation du fromage fabriqué à partir de mélanges de lait brebis-vache. Cette procédure a permis de détecter facilement une adultération par 5% de lait de vache dans le fromage Roquefort affiné pendant une période variant de 10 jours à 5 mois.*

isoélectrofocalisation / PhastSystem® / Roquefort / immunoblotting

INTRODUCTION

Bovine milk-adulterated Roquefort ovine cheese has been detected by electrophoresis on polyacrylamide gel at alkaline pH of the casein fraction (Assenat, 1967). Recently, bovine milk in adulterated Roquefort cheese has also been identified by gel isoelectric focusing of the para-κ-casein components with a sensitivity of about 5% (Mauriello *et al,* 1989). In cheese older than 1 month an interfering peptide from ovine milk comigrating with bovine para-κ-casein was found and a positive response for bovine milk was often given for pure ovine cheese (Addeo *et al,* 1990a). Bovine and ovine milk in mixtures can also be determined by gel isoelectric focusing detecting γ_2-caseins from the 2 species (Krause and Belitz, 1985). In order to amplify the response for bovine milk, the method was improved treating cheese proteins with plasmin prior to the electrophoretic analysis (Addeo *et al,* 1990b). In addition to the conversion of the β-casein into γ-caseins, a simplification of the casein pattern was realized together with the hydrolysis of some peptides migrating into the γ_2-casein area.

In a previous paper (Moio *et al,* 1990) we reported the development of a fast, sensitive procedure for the detection of bovine milk in ovine, caprine and water buffalo milk or cheese using small polyacrylamide gels containing urea on a fast electrophoresis system. In this paper, a method for a rapid evaluation of bovine milk in adulterated Roquefort cheese by PhastSystem® electrophoresis is described.

MATERIALS AND METHODS

Cheese preparation

Roquefort cheeses containing from 0–20% bovine milk, were prepared according to the standard procedure used in the Caves de Roquefort Society (France) (Assenat, 1985). Cheese samples were ripened for different periods of time (from 10 days to 5 months) and afterwards stored at –18 °C.

Isolation of cheese proteins

Freeze-dried cheese samples were defatted by extraction with diethylether in a Soxhlet apparatus and suspended in 9 mol/l urea containing 0.1% 2-mercaptoethanol at 1% final concentration (w/v). One μl of the resulting solution was applied at the anode side of the gel.

Isolation of pH 4.6 insoluble nitrogen fraction

A 6-g cheese aliquot was suspended in 60 ml distilled water, homogenized for 1 min with an Ultra-Turrax and kept in a water bath for 15 min at 90 °C. The suspension was centrifuged at 2 000 *g* for 10 min and the fat layer discarded. The precipitate was dissolved in 24 ml aqueous 7 mol/l urea, containing 0.1% 2-mercaptoethanol. The resulting suspension was centrifuged at 2 000 *g* for 10 min, the fat residue removed and the solution filtered on a paper filter (Whatman n 40). The clear solution was treated with 80 ml of 0.1 mol/l acetic acid–sodium acetate buffer at pH 4.6.

Finally, the precipitate, recovered by centrifugation at 2 000 *g* for 10 min at 10 °C, was washed twice with 20 ml distilled water. The wet product was either freeze-dried or squeezed for subsequent treatment or analysis.

Plasminolysis of cheese proteins

An aliquot (25 mg) of freeze-dried pH 4.6 insoluble nitrogen fraction was suspended in 500 μl of 0.05 mol/l sodium tetraborate buffer at pH 9, homogenized for 1 min with a Vortex and kept for 15 min at room temperature. 500 μl of protein solution was treated with 12 μl of bovine plasmin suspension (Boehringer, Mannheim, Germany) and incubated at 37 °C for 60 min. The reaction

was stopped by adding 500 µl 24% (w/v) TCA and the crude precipitate was collected by centrifugation at 2 000 *g* for 5 min. Finally, the precipitate was dissolved in 200 µl of 9 mol/l urea and 1 µl was applied at the anode side of the gel for subsequent gel isoelectric focusing.

Polyacrylamide gel isoelectric focusing by PhastSystem®

Fast isoelectric focusing (FIEF) of cheese proteins was performed in small ultrathin polyacrylamide gels containing 2% ampholytes and 7 mol/l-urea according to the procedure adapted for PhastSystem by Moio *et al* (1989). A linear gradient in the pH range of 5–7.7 was obtained by mixing Ampholine (LKB, Bromma, Sweden) pH 5–7 and Pharmalyte (Pharmacia, Uppsala, Sweden) pH 6.7–7.7 in a ratio of 1:5 (v/v). FIEF was carried out on the PhastSystem apparatus, using the running program reported in table I.

The gels were stained with Coomassie brilliant blue G-250 according to Krause *et al* (1988) and the silver staining procedure of Heukeshoven and Dernick (1988).

Densitometry

Densitometric tracing of minigels was performed with an LKB 2202 UltroScan Laser Densitometer (LKB) interfaced with an Apple IIe integrating computer.

Blotting and protein identification by enzymatic immunoassay

After polyacrylamide gel isoelectric focusing with a PhastSystem® apparatus, protein bands were immediately transferred onto nitrocellulose (NC) membrane (0.45 µm, Trans-Blot, Bio Rad, Richmond, CA, USA) by capillary blotting. The NC membrane, equilibrated for 10 min in 50 mmol/l NaH_2PO_4 and 0.9% NaCl (PBS, pH 7.2), was carefully layered onto the gel surface and covered with 3 sheets of Whatman 3 MM filter paper saturated with PBS at pH 7.2, then 3 additional sheets of dry Whatman 3 MM and finally a 3-mm thick glass plate weighted with about 3 g/cm^2. During the transfer (25–30 min) the temperature of the separation bed was microprocessor-controlled at 30 °C. The immunodetection of transferred antigens was performed according to the procedure described by Tsang *et al* (1983).

RESULTS AND DISCUSSION

In order to destroy the enzyme activity naturally occurring in the cheese and able to hydrolyze casein during incubation with

Table I. Voltage program for running fast isoelectric focusing of the γ_2-casein fractions on small polyacrylamide gel containing urea in the pH gradient 5–7.7.
Voltages utilisés pour l'isoélectrofocalisation des fractions de caséines (γ_2 gel de polyacrylamide-urée; gradient de pH 5–7,7).

Step	*Voltage (V)*	*Current (mA)*	*Power (W)*	*Temp (°C)*	*Vh*
Prefocusing	1 200	2	2.5	15	75
Sample application	200	2	2.5	15	15
Focusing	1 200	5	2.5	15	710

Sample applicator installed after 75 Vh and removed after an additional 15 Vh (total 90 Vh).
Applicateur d'échantillon déposé après 75 Vh et enlevé après 15 Vh supplémentaires (total 90 Vh).

plasmin, it was decided to treat the cheese suspension at 90 °C for 15 min. Afterwards, the cheese casein fraction was isolated by isoelectric precipitation. The efficiency of the treating procedure was controlled by incubating casein at pH 8.6 for a long period of time. No proteolysis was detected, whilst treatment with plasmin brought about the disappearance of some casein bands migrating in the γ_2-casein area (results not shown). Therefore, the use of the casein fraction is rather better than the total cheese protein as a substrate for plasmin action. Figure 1 shows the electrophoretic patterns on small plates produced by polyacrylamide gel isoelectric focusing of cheese samples containing 5 and 10% bovine milk ripened for periods ranging from 10 days to 5 months after *in vitro* plasmin action. Using this enzyme as a signal amplifier for γ_2-caseins, up to 5% bovine milk was clearly detected in the cheese sample adulterated with bovine milk, irrespective of age (slots 1A, 1B, and 1C, fig 1). Due to the high sensitivity of the silver staining procedure, mainly in the case of gel overloading, a faint band with pI value similar to γ_2-casein was observed which did not have an intensity higher than 0.3% (fig 1A). Since development with silver staining is similar to the photographic process, the coloration of the minor bands can be artificially increased with length of development time. Therefore, in order to detect under 5% concentrations of bovine milk using the silver stain, it is better to use standard cheese samples with known compositions of bovine and ovine milk together with the samples at unknown level of bovine milk. On the other hand, when the estimated content of bovine milk is higher than 5%, the gel can be stained with Coomassie blue and the evaluation of bovine milk obtained directly from the densitometric figures corresponding to the percentage of bovine γ_2-casein in the total γ_2-casein without the use of standard samples. In order to evaluate the relative content of γ_2-caseins in the mixtures, the intensity of the γ_2-casein bands was measured using densitometry. Table II shows the percentage of bovine and ovine milk in mixtures calculated for a Roquefort cheese of known composition. The figures found are in good agreement with the true values.

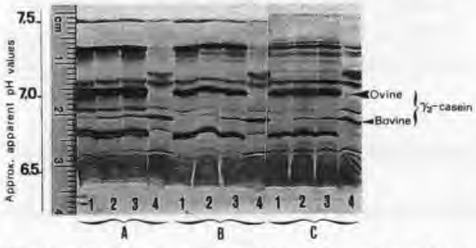

Fig 1. FIEF of pH 4.6 insoluble nitrogen fraction isolated from Roquefort cheese containing bovine milk after plasmin action (for the procedure refer to the *Methods* section). 1, 2, 3 and 4 = 0%, 5%, 10% and 100% bovine milk respectively. A, B and C = 10 days, 1 month and 5 months respectively. Silver staining.
Profils de FIEF de la fraction azotée précipitée à pH 4,6 des fromages de Roquefort après hydrolyse par la plasmine. 1, 2, 3 et 4 = 0%, 5%, 10% et 100% vache. A, B et C = 10 jours, 1 mois et 5 mois d'affinage. Coloration à l'argent.

When the total cheese proteins are analyzed without the addition of plasmin, a band specific for bovine milk, the R band, was observed on the alkaline side of the pH gradient (fig 2) irrespective of cheese age. The origin of this band was established using an immunochemical test in which rabbit antisera were raised against bovine α_{s1}-, α_{s2}-, β- and κ-casein. Figure 3 shows the immunoblotting print relative to 4-month-old Roquefort cheese samples using anti-β-casein sera. The R band is immunospecifically stained in all cheese samples containing bovine milk except for one ovine cheese. Furthermore, the R band was not recognized by the anti α_{s1}-, α_{s2}- and κ-casein immunosera (results not

Table II. Evaluation of the amount of bovine milk in Roquefort cheese samples reported in figure 1.
Evaluation de la proportion de lait de vache dans les échantillons de fromage de Roquefort reportés dans la figure 1.

Lane	*Theoretical values (%)*	*Values found (%)*
1A	0	0.3
2A	5	6 ± 1
3A	10	12 ± 2
4A	100	100
1B	0	0
2B	5	7 ± 2
3B	10	11 ± 3
4B	100	100
1C	0	0
2C	5	5 ± 0.5
3C	10	9 ± 1
4C	100	100

Values are the averages of 3 determinations with the standard deviations.
Les valeurs sont la moyenne de 3 déterminations avec l'écart type.

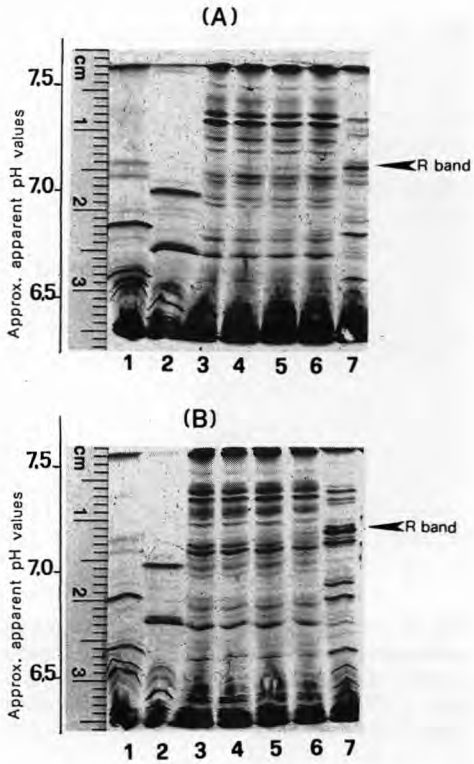

Fig 2. FIEF of cheese proteins from Roquefort cheese at 2 stages of ripening (A) = 1 month, (B) = 5 months. (1) and (2) reference γ-casein fraction obtained by action of plasmin on whole bovine and ovine casein, respectively. (3), (4), (5), (6) and (7) Roquefort cheese samples containing 0, 5, 10, 20 and 100% bovine milk, respectively. Coomassie brilliant G-250 staining.
Profils de FIEF des protéines du fromage Roquefort à deux stades d'affinage (A) = 1 mois, (B) = 5 mois. (1) et (2) caséines γ préparées par action de la plasmine sur une caséine de référence isolée à partir du lait de vache et de brebis. (3), (4), (5), (6) et (7) : échantillons de fromage Roquefort contenant 0, 5, 10, 20 et 100% de lait de vache. Coloration avec Coomassie Brilliant G-250.

shown). Therefore, it can be concluded that the peptide corresponding to the R band arises from bovine β-casein. Thus the R band might represent an additional marker of bovine milk. Since this component is not derived from the action of plasmin in cheese, no pre-treatment of the cheese with plasmin is required to detect it. This peptide might have arisen from limited hydrolysis of β-casein by the action of mould enzymes. Figure 4 shows 3 regression curves relative to Roquefort cheeses of different ages obtained by plotting the intensity of the R band against the percentage of bovine milk in the mixtures. A linear relationship was found between the intensity of the bovine R band and the percentage of bovine milk in mixtures (from 5–20% bovine milk). However, the R band content depends on the ripening period of the cheese reaching a maximum value at 4

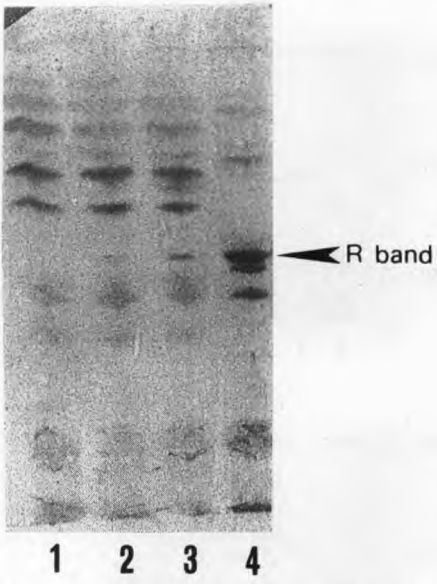

Fig 3. Immunodetection with rabbit polyclonal anti-bovine β-casein sera of Roquefort cheese caseins transferred to nitrocellulose membrane after FIEF on polyacrylamide gel. The samples contain different amounts of bovine milk (1:0%; 2:5%; 3:10%; 4:100%).
Immunodétection utilisant des anticorps polyclonaux contre la caséine β bovine. Application sur la fraction caséique du Roquefort après transfert sur une membrane de nitrocellulose du profil de focalisation sur gel de polyacrylamide. Les échantillons contiennent des quantités croissantes de lait de vache (1:0%, 2:5%, 3:10%; 4:100%).

months ripening. Afterwards, it decreases sharply so that the quantitative assay of bovine milk, based on the evaluation of the R band, becomes unreliable. Since no cheese over 5 months of age and containing known levels of bovine milk was examined, further work is needed to follow the degradation of β-casein in older Roquefort cheese.

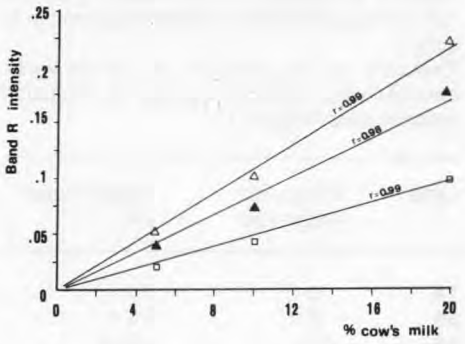

Fig 4. Calibration curves for 1- (▲, Y = 0.008X – 0.12), 4- (Δ, Y = 0.011X – 0.01) and 5- (□, Y = 0.005X – 0.04) month-old Roquefort cheese samples containing 5–20% bovine milk. The intensity of bovine R band is plotted against the percentage of bovine milk in the mixture.
Représentation graphique du dosage du lait de vache dans le Roquefort de 1 mois (▲, Y = 0,008X – 0,12), 4 mois (Δ, Y = 0,011X – 0,01) et 5 mois (□, Y = 0,005X – 0,04) d'affinage. Le graphique représente la variation de l'intensité colorimétrique de la bande R en fonction du pourcentage de lait de vache dans le mélange.

CONCLUSION

Bovine γ_2-casein was distinctly separated from the homologous ovine counterpart in Roquefort cheese using the PhastSystem® apparatus and minigels containing urea and ampholytes. In evaluating this component, levels of bovine milk as low as 5% were easily detected in 1–5 month old Roquefort cheese. However, in order to obtain a clear electrophoretic pattern in the γ-casein region, it was essential to use the insoluble nitrogen fraction of the cheese at pH 4.6 as a substrate for plasmin action. In the case of analysis of the total nitrogen fraction electrophoretic patterns more complex than those exhibited by the pH 4.6 insoluble nitrogen fraction were obtained.

When analysing the casein fraction no bands co-migrating with bovine γ_2-caseins were detected, and a clear separation of bovine γ_2-casein from the interfering peptides was achieved. In addition to γ_2-casein a marker for bovine milk was found in Roquefort cheese ripened for more than 1 month, made with mixture of ewe and cow milk. It derives from β-casein hydrolysis during ripening. Since it was not detected in hard-pressed ovine cheese such as Pecorino, within 12 months of ripening, the authors suggest that the novel marker for bovine milk arises from the limited hydrolysis of β-casein by a mould enzyme.

In conclusion, it is confirmed that the detection of bovine milk in Roquefort cheese provides reliable quantitative results when γ_2-casein is evaluated and only semi-quantitative results on cheese samples over 1 month old in the case of the R band. The main advantage of this system is the improved resolution between γ_2-casein fractions and the short time required for analysis.

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