Electrophoretic isolation of β-casein and optimization of a radial immunodiffusion test for bovine milk quality control

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A B S T R A C T

Beta-casein (β-CN) is a major dairy protein subject to preferential degradation during storage. Our study aimed to isolate β-casein from bovine milk under dissociative conditions by the electrophoretic technique using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and optimize an immunochemical technique such as Mancini radial immunodiffusion in order to monitor the evolution of this milk protein during storage. For this purpose, a series of physicochemical and bacteriological analyses revealed conformity with international standards. This allowed us to use quality milk for more detailed studies of proteins, in particular β-casein, which was selected as a marker of milk protein quality. The total casein of mixed milk samples was isolated and then submitted to electrophoretic separation. Various combinations of acrylamide and bisacrylamide were used and the one corresponding to the ratio 8 g / 230 mg was retained. A total casein deposit of 833 μg provided an adequate β-casein concentration (300 μg) for consistent animal immunization. Thirty *Wistar* rats were subjected to an immunization protocol for 35 days. The obtained antisera were used to optimize the Mancini technique and to assay β-casein in our samples at different storage times ranging from 3 h to 69 h/+4°C. A decrease in β-casein content from 0.85 mg ml^{-1} to 0.32 mg ml^{-1} was detected. These results demonstrate the vulnerability of β-casein at low temperatures and provide information on the origin of milk and its application for dairy or cheese production. In addition, our study confirms the usefulness of immunochemical techniques such as the Mancini test in the determination of β-casein.

Keywords: dairy, β-casein, electrophoretic technique, radial immunodiffusion, storage, quality.

И З В О Д

Бета-казеин (β-CN), један од главних протеина млека, подложан је деградацији током чувања млека. Циљ овог истраживања је да се изолује β-казеин из крављег млека у условима дисоцијације помоћу електрофорезе на натријум-додецил-сулфату у полиакриламидном гелу и да се оптимизује имунохемијска техника, тј. Манцинијева радијална имунодифузија, ради праћења овог протеина млека током складиштења. Спроведене физичко-хемијске и бактериолошке анализе показале су усклађеност с међународним стандардима. То нам је омогућило да квалитетно млеко употребимо за детаљнија испитивања протеина, нарочито β-казеина, који је употребљен као маркер квалитета протеина у млеку. Из мешаних узорака млека изолован је укупни казеин и подвргнут електрофоретској сепарацији. Коришћене су различите комбинације акриламида и бисакриламида, а задржана је она с односом 8 g / 230 mg. Талог укупног казеина у количини од 833 μg омогућио је довољну концентрацију β-казеина (300 μg) за доследну имунизацију животиња. Тридесет пацова соја *Wistar* подвргнуто је имунизацији током 35 дана. Добијени антисеруми употребљени су за оптимизацију Манцинијеве технике и испитивање β-казеина у узорцима млека у различитим периодима чувања млека од 3 h до 69 h/+4°C. Утврђено је смањење садржаја β-казеина од 0,85 mg ml^{-1} до 0,32 mg ml^{-1}. Ови резултати показују осетљивост β-казеина на ниским температурама и пружају информације о пореклу млека и његовој примени у производњи млечних производа или сира. Осим тога, наше истраживање потврђује корисност имунохемијских техника, као што је Манцинијев тест, у одређивању β-казеина.

Кључне речи: млечни производи, β-казеин, техника електрофорезе, радијална имунодифузија, чување, квалитет.

1. Introduction

Algeria is the biggest consumer of milk in the Maghreb, with approximately 120 L/year/inhabitant (El Hassani, 2013). This food represents a major part of the diet food ration of Algerians and provides the largest part of proteins of animal origin (Senoussi, 2008). Cow's milk is a liquid food rich in nutrients such as proteins, calcium, magnesium and phosphorus (Hanusová et al., 2010). Because of their nutritional properties, cow's milk proteins are among the most valuable components of the human diet. Many of these proteins are considered in evaluating milk quality in many dairy industries but also in dairy cow selection (Franzoi et al., 2019).

Caseins are among major proteins in milk. They are subdivided into four groups, namely: alpha-casein 1 (α S1), alpha-casein 2 (αS2), beta-casein (CSN2) and kappa-casein (CSN3) (Volkandari et al., 2017). Beta-casein constitutes 45% of the total caseins in cow's milk (Massella et al., 2017). Beta-casein is one of the proteins involved in the quality and composition of cow's milk. It influences the cheese formation process, cheese ripening time and the firmness of curdled milk (Amalfitano et al., 2019; Saha et al., 2020).

Milk samples used in this study were collected at the Giplait dairy in Sidi Bel-Abbés, Algeria, and the milk received daily can remain a long time in the tanks at a low temperature before use. The tank allows the milk to be stored at a temperature that ensures its proper conservation until it is collected by the dairy. In concrete terms, the temperature of the milk is about 38°C when it leaves the cow's udder. When it arrives in the tank, it is immediately cooled to 4°C to limit the development of micro-organisms. The dairy comes to collect the milk stored in the tank, with an isothermal tanker. The milkman takes a sample for quality control and then connects a pump from the tanker directly to the tank. Then , the milkman transports the milk to the dairy (3 hours between the collection of the tank at the farm and the delivery to the dairy), where it is controlled and then conditioned (for liquid milk) and transformed into dairy products (cream, butter, cheese, yoghurt, dairy desserts...) in the hours following its arrival.

The time between milk collection and milk delivery can become significant when milk is retained by providers who also store it at low temperatures before delivery. These conditions can become compromising for milk protein quality. The targeting of this product for cheese production may become economically unsuitable.

In this context, the main objective of our study was the isolation of β-CN by electrophoretic techniques and the optimization of an immunochemical test, Mancini radial immunodiffusion test, in order to determine the degree of casein proteolysis as a function of the storage time of mixed milk.

2. Materials and methods

2.1. Origin and provenance of the milk

Samples of mixed bovine milk (morning milking + evening milking) from healthy Holstein cows located at farms in Sidi Bel Abbés were properly collected, supplemented with antibacterial additives (solution of thiomersal, 0.005 %, p/v) and immediately transported to the laboratory (in a cool box), where it was analyzed.

2.2. Milk quality control

2.2.1. Physico-chemical control

The physico-chemical study focused on the determination of acidity, fat, density, total dry matter (TDM) and defatted dry extract (DDE) by using the AFNOR protocols (1986).

2.2.2. Microbiological control

According to the Official Journal of the Algerian Republic (JORAN°39, 2017), milk microbiological analyses were carried out: Aerobic germs, coliforms, fecal coliforms, *Staphylococcus aureus* and phosphatase. The latter were performed on selective media. Aerobic germs were determined on a Plate Count Agar (PCA) medium and the other tests were performed on VRB as a solid medium and VBL as a liquid medium.

2.3. Study of the electrophoretic properties of the isolated caseins

2.3.1. Preparation of total caseins

Total caseins were separated from raw milk by precipitation at pH 4.3, by the addition of 1N HCl. After heating for 10 min at 30 to 35°C temperature, caseins were recovered by centrifugation at 4500 ×g for 30 min (4°C) (Alim et al., 2005).

The obtained precipitate was washed with distilled water and centrifuged one more time. This washing process was repeated three times to remove soluble whey proteins and non-protein constituents such as lactose and minerals (Wakabayashi et al., 2006).

The obtained caseins were resolubilized at pH 7 by adding 1N NaOH and the second precipitation was performed (at pH 4.6). The caseins were finally resuspended in a sufficient amount of distilled water and resolubilized at pH 7 (1N NaOH). For better conservation, the prepared bovine caseins were lyophilized and stored at -20°C until use.
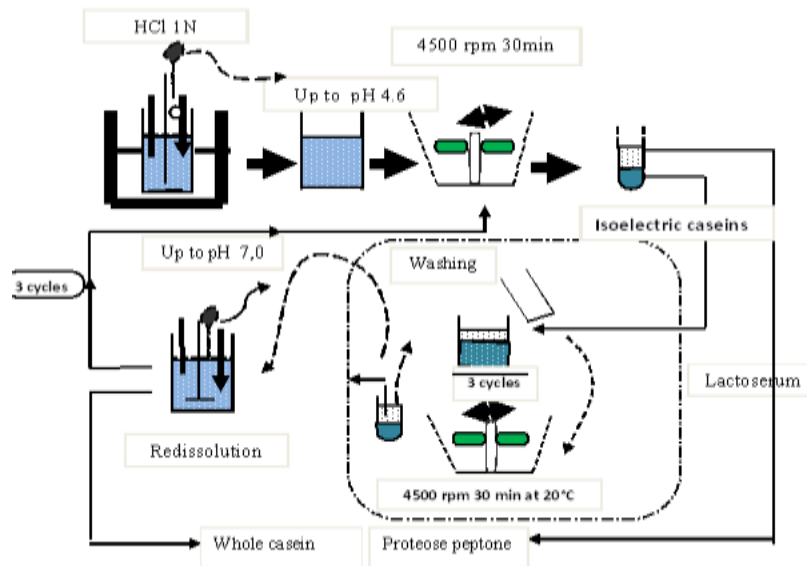
Figure 1. Total casein production process

2.3.2. SDS-PAGE electrophoresis

The isolated total caseins were characterized by electrophoresis (SDS-PAGE) on polyacrylamide gel in the presence of the denaturing agent Sodium Dodecyl Sulfate (SDS) (Laemmli and Fave, 1973).

The electrophoresis was performed in a vertical mini-Protean II slab gel apparatus (Bio-Rad Laboratories, California, USA) according to the following experimental conditions: separation gel: 30% of acrylamide, a temperature of 16°C maintained by a refrigeration system, voltage: 150–225 V, amperage: 25–45mA/1 mm, time: 5 hours/10cm.

A protein standard kit, containing bovine α-lactalbumin (14,200 Da), soybean trypsin (20,100 Da), bovine trypsinogen (24,000 Da), and Bovine carbonic anhydrase (29,000 Da), was used to calibrate the gel.

2.3.3. Immunochemical control of β-casein

2.3.4. Protocol for the production of anti β-CN antiserum

According to the protocol of Duddukuri et al. (2001), 30 Wistar strain rats (80 ± 5g) were immunized over a period of 35 days by subcutaneous administration in order to produce polyclonal antibodies (anti β-CN). The adjuvants used were in oily form of two types: Freund's complete adjuvant (FCA) (Sigma F5881, lot 093K832) used for primary immunization and incomplete Freund's adjuvant (IFA) (F5506, lot 025K810) used for booster injections (about every 2 weeks).

At the end of each electrophoresis performed, the bands corresponding to β-casein were carefully collected and added to 500 µl of PBS buffer. The solution was filtered through a 0.45µm microfilter.

Under these conditions, the addition of an equal amount of adjuvant (V/V) allowed the injection of an adequate and necessary amount of β-casein, in this case 300µg ml^{-1}, for the animal's immune response. On the 35th day of the experiment, the serum was collected and aliquoted, and then frozen at -20°C until use.

2.3.5. Radial immunodiffusion: Mancini test

The estimation of β-casein was performed by using the radial immunodiffusion technique of (Mancini, 1965). The serum (anti-β-casein) was mixed with an equal volume of 2% agar (pH 7.2) at 50°C (V/V); a volume of the mixture was transferred into Petri dishes.

For this purpose, undiluted antiserum and then diluted to 4/5, 3/5, 2/5 and 1/5 in PBS buffer was used. After solidification of the mixture, 3 mm diameter wells were formed. In each well, the stock solution of undiluted β-casein and β-casein diluted in PBS buffer at 0.25 mg ml^{-1}, 0.5 mg ml^{-1}, 0.75 mg ml^{-1} and 1 mg ml^{-1} was added. The incubation was carried out at 37°C for 72 hours. The optimal dilutions of each reactant (bovine β-casein, anti-β-casein) were determined.

The optimization of the Mancini technique (1965) allowed us to study the β-casein kinetics evolution as a function of storage time (3; 9; 27; 33; 69 hours/+ 4°C).

3. Results and discussions

3.1. Physicochemical parameters

The results illustrated in Table 1 showed conformity with the standards required by the AFNOR (French national organization for standardization), thus confirming that the milk was of excellent physico-chemical quality.

Table 1.

Physicochemical analysis of mixed milk

Parameters	Mixed milk	AFNOR standard
Acidity (°D)	17°	14–18°
Fat (g l^{-1})	33	34 and more
Density	1031.4	1030 and more
TDE (g l^{-1})	120	119 and more
DDE (g l^{-1})	87	87 and more

TDE: total dry extract; DDE: defatted dry extract

Table 2.

Microbiological analysis of mixed bovine milk

Control parameter (CFU/ml) Sample		Aerobic germs at 30°C	Coliforms	Fecal coliforms	*Staphylococcus aureus*	Phosphatase
Mixed milk		2.10^4	0.5	Absence	0.5	Negative
Standards	Standards m	3.10^4	01	00	Absent	Negative
	Standards M (Liquid environment)	9.10^4	30	00	Absent	Negative
	Standards M (Solid environment)	3.10^5	10	00	Absent	Negative

N: Number of germs per ml

m: the limit level under which the product is considered to be of satisfactory quality; all results equal or lower than this criteria are considered satisfactory

M: acceptability limit over which the results are not considered satisfactory and the product is considered toxic.

M= 10m in solid medium

M= 30m in liquid.

If N<m, the result is satisfactory.

If m <N<M, the result is acceptable.

If N>M, the result is not satisfactory and therefore the milk does not conform to the standards.

The microbiological analysis of the mixed bovine milk showed that the results conformed to the standards and were satisfactory.

3.3. Protein electrophoretic profile

The physicochemical and microbiological analyses allowed us to use milk quality for the study of protein electrophoretic properties, in particular β-casein selected as a marker of milk protein quality.

3.4. First SDS-PAGE analysis

The first separation experiment of total casein is illustrated in Figure 2. A combination of acrylamide and bisacrylamide concentrations resulted in a rapid migration of all casein molecules, in this case αs-casein, β-casein and κ-casein. In fact, the 35% acrylamide / 2% bisacrylamide ratio was not sufficient to provide appropriate porosity for the clear and early separation of these proteins
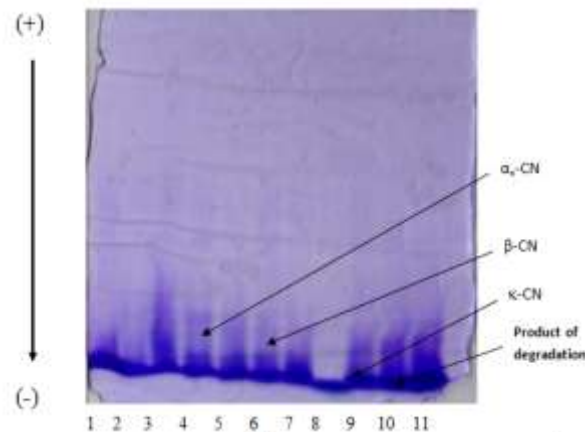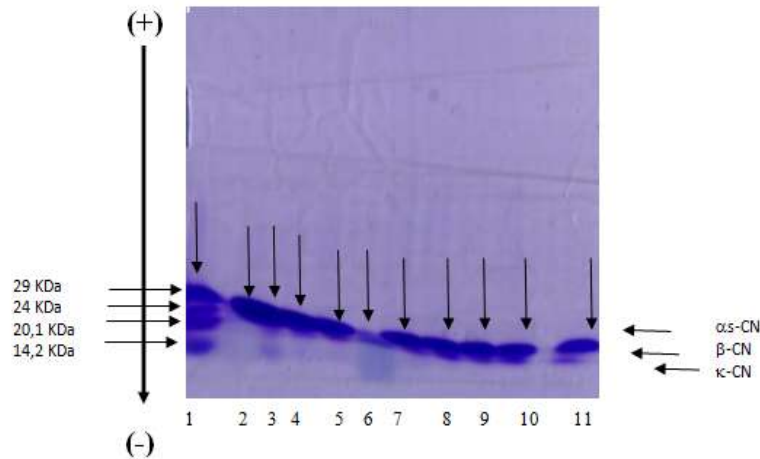

Figure 2. Electrophoresis of total casein on polyacrylamide gel (35%/2%) under dissociative conditions

3.5. Second SDS-PAGE analysis

This second analysis used a combination of 30% acrylamide / 0.8% bisacrylamide with a 20% SDS concentration in the separation gel. We considered that these proportions would provide better resolution. In fact, the bands corresponding to the different caseins are visible, and the same applies to the proteins of the used markers (Figure 3).
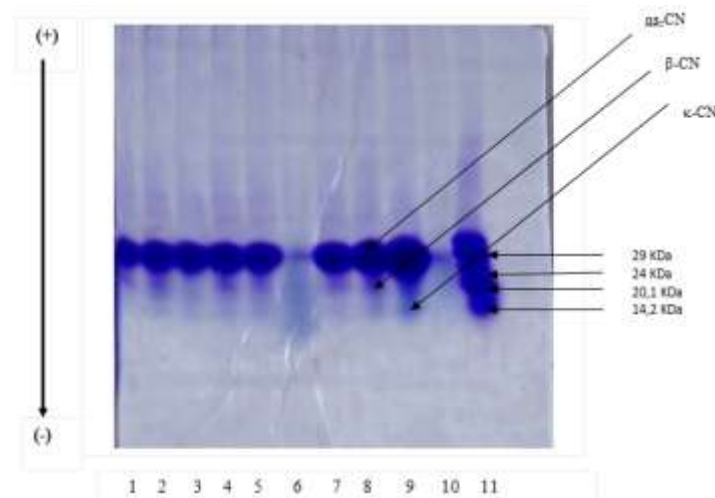

Figure 3. Electrophoresis of total casein on polyacrylamide gel (30%/0.8 %) under dissociative conditions

Well 1 showed an appreciable resolution of molecular weight markers, with the identification of the band corresponding to bovine carbonic anhydrase (29 KDa), bovine trypsinogen (24 KDa), soybean trypsin inhibitor (20.1 KDa) and bovine α-lactalbumin (14.2 KDa). Well 2 showed a broad band that can be assimilated to the different caseins that remained indissociable. This band corresponds to the total casein of milk. The three visible bands in well 3 are clearly identifiable with the three molecular weights of the marker; and also with the different caseins in the milk. For this sample, it is a question of a defective dissociation of the caseins. The band corresponding to α_s-CN is very dense, indicating a high concentration of this protein; this is not the case for β-CN and κ-CN, whose bands are less concentrated and for the different deposits.

3.6. Third SDS-PAGE analysis

This analysis was performed using 8g of acrylamide and 230 mg of bisacrylamide in Tris-HCl buffer pH 8.9. The bands of different caseins are visible; as are the proteins of the used markers (Figure 4). The studied milk showed bands that were clearly visible and assimilated to caseins, α, β and κ. There are no bands corresponding to the degradation products of the latter. This explains the excellent processing conditions of the raw material and the conservation of the total casein.
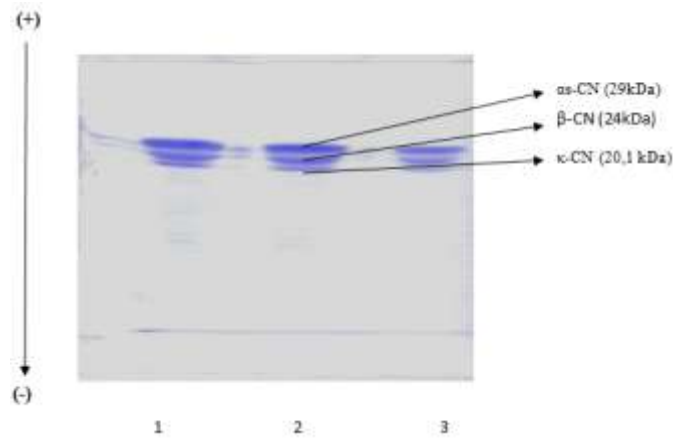

Figure 4 . Electrophoresis of total casein on polyacrylamide gel (8 g/230mg) under dissociative conditions

3.7. Fourth electrophoretic analysis

Appropriate acrylamide/bisacrylamide combinations (8g/230g and a casein deposit corresponding to the concentrations that allow consistent immunization of the animals (833μg of whole casein) were performed. The obtained bands attest the purity of β-casein and the facility of its sampling (Figure 5).


Figure 5. Polyacrylamide gel electrophoresis of total casein at 833μg (8g/230mg) under dissociative conditions.

3.8. Evaluation of β-casein content by the Mancini radial immunodiffusion technique

Through the optimization of the Mancini technique, we noticed that only the use of undiluted antiserum (anti-β-casein) for different antigen concentrations (β-casein solution) provides positive results (precipitation ring formation). The calibration curve allowed us to determine the concentration of the different samples to be analyzed. $d^2 = f\ ([β\text{-}CN])$ (Figure 6).
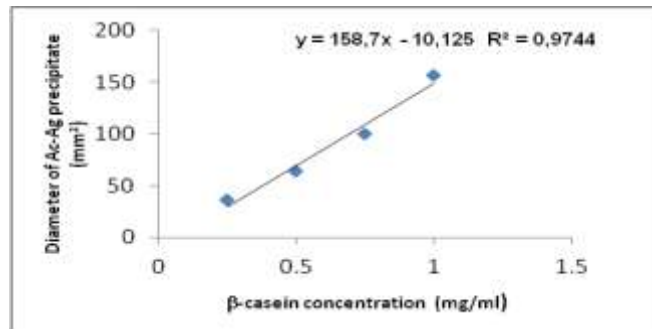
Figure 6. Calibration curve for the β-CN concentration calculation

This procedure for antigen obtention as well as the technique adopted for the immunization effectively provided positive results regarding the immunogenicity of β-casein demonstrated by the Mancini technique.
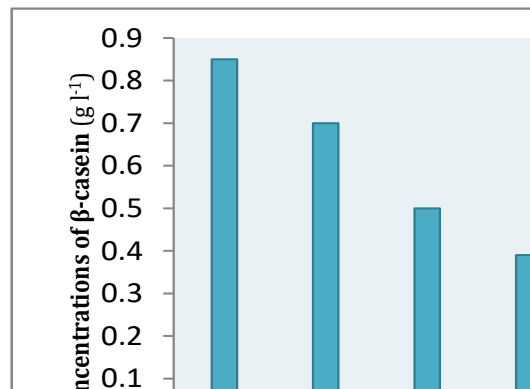
Figure 7. β-casein concentration in milk according to storage time

The results confirm β-casein degradation in the mixed milk, which after 69 hours of storage at +4°C also showed a reduction of 37% (from 0.85mg ml-1 after 3h to 0.32mg ml-1 after 69h) (Figure 7).

According to Croguennec (2008), milk refrigeration causes the solubilization of colloidal calcium phosphate and β-CN (micelle disorganization). These modifications are partially reversible after returning to the renneting temperature: β-CN, which is preferentially located at the center of the native casein micelle, tends to be positioned at its surface after a period at low temperature (24 to 72 hours at +4°C).

Its localization blocks the rennet attack with a consequent increase in hydrolysis time. The presence of β-CN on the surface of casein micelles also reduces the aggregation rate by creating steric interference to the closeness of the micelles: aggregation time and gelification increase. In addition, β-CN also has the ability to auto-associate during the temperature increase and to form β-CN micelles. These micelles are not involved in protein matrix formation. In addition, β-CN can be hydrolyzed during refrigeration by proteases including plasmin.

The hydrophilic and soluble peptide fragments of β-CN are removed during serum expulsion. This results in a reduction in gel firmness and cheese yields.

Some authors have determined the real cleavage sites of caseins by various enzymes, including plasmin. This has been done on casein, which, when hydrolyzed by plasmin, provides protease-peptones and gamma caseins (Bars and Gripon, 1989).

The centralization of the dairy industry has promoted milk storage between +4°C and +7°C, creating problems due to the selection of psychrotrophic bacteria; in addition to the degradation by these bacteria, there are alkaline proteases. At high temperatures, plasmin and β-CN are bound to the surface of the micelles, which prevents proteolysis. When milk is stored at +4°C for 48 hours, the concentration of γ-CN (degradation product of β-CN) increases by 25%. This protease has a specificity similar to that of trypsin and cleaves peptide bonds of the arg-x,lys-x type. Beta-CN, which contains 11 lysines and 4 arginines, can therefore be divided into several fragments during proteolysis (Reimerdes and Herlitz, 1979).

In fact, there are only 3 main cleavages: lys28-lys29, lys105-lys106, and lys107-glu108, resulting in three major C-terminal fragments, which are easily detected by electrophoresis and which correspond to gamma1 (29-209), gamma 2 (106-206) and gamma 3 (108-209) caseins. The N-terminal casein complements represent the two main acid-soluble and thermostable compounds of the protein-peptone fraction and are only found in whey.

In fact, Groves et al. (1962), Gordon et al. (1973) and Kaminogawa and Yamauchi (1972), have established a relationship between gamma caseins and beta caseins based on the results of amino acid analysis, determination of molar mass, peptide cards and COOH-terminal studies. They concluded that gamma caseins were fragments of different sizes, proteolysis residues, representing the C-terminal part of β-CN: γ1 (29-209), γ2 (106-209) and γ3 (108-209) Eigel et al. (1984).

Protease-peptones constitute the minor protein fraction of whey. They remain soluble after heating to 95°C and acidification to pH 4.5. Three components 3, 5 and 8 are identified according to their increasing electrophoretic mobility. The three constituents have been designated by the abbreviations pp3, pp5 and pp8, the terms adopted by the American Dairy Science Association (Alais and Linden, 1997).

Currently, the three major fragments identified are from beta-casein. The numerous cleavage sites in β-casein correlate with the content of this casein degraded during storage of recombined milk and that of mixtures. Gamma-caseins and peptone proteases remain to be quantified by appropriate techniques , to measure differently the extent of the degradation of milk and consequently its orientation to dairy or cheese production.

4. Conclusions

A proportional relationship was found between milk storage time and β-CN degradation. The applied immunochemical technique, which is both reproducible and performant because it is based on the specific reaction of antigens and antibodies, can be used to control the quality of the milk received during the year and allow the quality control of this product in order to remunerate the provider by protein content and not by milk volume.

Acknowledgment

Declaration of competing interest

The authors declare that there is no conflict of interest.

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