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Evaluation of proteolysis in Parmesan cheese using electrophoresis and HPLC*

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Abstract – The proteolytic changes occurring during the ripening of Parmesan cheese were studied using urea-polyacrylamide gel electrophoresis (urea-PAGE) of caseins, HPLC analysis of free amino acids (FAA) and Kjeldahl determination of soluble nitrogen fractions. An electrophoretic ripening index for the evaluation of proteolysis in Parmesan cheese was established. The separation of caseins by alkaline PAGE (12 % T, 2.6 % C, pH 8.9, 5 M urea) was followed by the densitometric analysis of the γ - and β -casein fractions. The relationship between the resulting coefficients (γ -Cn/ β -Cn) and the age of reference samples of Original Italian Grana Padano (6–22 months) was linear up to 15 months, allowing an evaluation of the extent of proteolysis and therefore a deduction of the age of the Parmesan samples analysed. Threshold levels (γ -Cn/ β -Cn) were proposed for the verification of the required age of Parmesan cheese. The coefficients (γ -Cn/ β -Cn) as well as the β -casein content of two additional series of references and of 117 commercial Parmesan samples are presented. Commercial Parmesan samples retailed as a loaf or as prepacked slices were generally found to fulfill the requirements concerning endoproteolytic changes during ripening. However, many grated Parmesan samples taken from retail outlets in Austria showed poor quality, which was probably due to the adulteration with products with low proteolysis (e.g., cheese rind, very young cheese). HPLC analysis of FAA was also used to characterize the ripening process. Although FAA content of reference samples of Grana Padano showed a very high degree of variability, a distinct increase of FAA content could be observed during the ripening period. However, two series of reference samples, which had completely different electrophoretic casein patterns, could not be distinguished by HPLC analysis of the total amount of FAA. As an experiment, two loaves of Parmesan cheese were removed from the ripening room at an age of 4 and 2 months and were subsequently stored at 6 °C for 18 months. During the storage period, endoproteolytic changes were slowed down and breakdown of α_{S1} - and β -casein as well as the accumulation of degradation products proceeded at a reduced rate, and could be detected using urea-PAGE casein patterns, but not by HPLC analysis of FAA content. In consequence of these findings, no accurate evaluation of the age of commercial Parmesan samples was possible by means of the total amount of FAA. Since Kjeldahl determination of WSN and HPLC analysis of FAA content give insufficient information about the distinct endoproteolytic changes, which were found to be typical for Parmesan cheese, urea-PAGE of the casein fraction has to be done to enable

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the evaluation of the extent of proteolysis and therefore the deduction of the age of commercial Parmesan samples analysed. © Inra/Elsevier, Paris

Parmesan cheese / proteolysis / electrophoresis / free amino acid / soluble nitrogen

Résumé – Évaluation de la protéolyse du fromage parmesan par électrophorèse et HPLC. La protéolyse du parmesan a été suivie au cours de l'affinage par électrophorèse (urée-PAGE) des caséines, analyse par HPLC des acides aminés libres et détermination par Kjeldahl des fractions azotées solubles. L'analyse des profils protéiques obtenus par électrophorèse a permis d'établir un index d'affinage grâce auquel l'âge des échantillons de fromages du commerce peut être estimé. L'utilisation de cet index a en outre permis de montrer que beaucoup de parmesan vendu sous forme de râpé était de mauvaise qualité. L'analyse des acides aminés libres ne permet pas en revanche de déterminer correctement l'âge du fromage. © Inra/Elsevier, Paris

fromage parmesan / protéolyse / électrophorèse / acide aminé libre / azote soluble

1. INTRODUCTION

Parmesan cheese has considerable commercial importance, therefore several methods have been reported to control quality and authenticity of Parmesan. One of the prerequisites established by production standards for Parmigiano-Reggiano, Grana Padano and other Italian hard cheese varieties labelled as Original Italian Parmesan Cheese is the minimum age of 12 months [1, 12]. In most cases, attention has been focused on the parameters that quantify and characterize proteolysis occurring during cheese ripening [9].

The degradation of the casein fractions proceeds rapidly during the first 8–10 months of ripening, slowing down considerably during further maturation of Parmesan cheese. This has been shown by following the variations in soluble nitrogen with respect to total nitrogen over time [6, 7, 29].

The changes in insoluble proteins have been studied using electrophoretic techniques [9, 22, 24]. It has been shown that β -casein is hydrolysed very rapidly during the first months of ripening, disappearing after approximately one year [1, 6]. Consequently, the γ_2 - and γ_3 -caseins continue to increase, and γ_1 -casein tends to decrease concomitantly, which confirms the impor-

tant role of plasmin in maturation [15, 23]. Due to manufacture technology, this indigenous milk proteinase remains active and contributes to proteolysis during the ripening of Parmesan cheese [14]. Using fast atom bombardment-mass spectrometry, it was also found that the majority of oligopeptides arises from β -casein [2–4].

In Parmesan cheese, the total amount of free amino acids (FAA) rapidly reaches higher levels than in other cheeses [7, 17–19]. The relative percentages of certain FAA were used to establish useful chemometric models for evaluating authenticity of Parmigiano-Reggiano [25, 26]. Based on the relative amounts of individual FAA, a two-dimensional chemometric model was established for distinguishing traditional Grana Padano from similar cheeses, although great differences were observed among the patterns of samples with the same ripening period but produced by different dairies [27].

The purpose of the present study was: i) to study proteolytic changes occurring during the ripening of reference samples of Parmesan cheese using urea-PAGE of caseins, HPLC analysis of FAA and Kjeldahl determination of soluble nitrogen; ii) to establish an electrophoretic ripening index for the evaluation of the extent of proteoly-

sis and the deduction of the age of Parmesan cheese, and to analyse commercial Parmesan samples with respect to this proteolysis index; and iii) to determine proteolysis in two loaves of Parmesan, which were removed from the ripening room at an age of 4 or 2 months and were subsequently stored at 6 °C for 18 months, and to study the effect of this storage period on the endoproteolytic changes, which were found to be typical for Parmesan cheese ripened according to the traditional technology.

2. MATERIALS AND METHODS

2.1. Cheese samples

Reference samples ($n = 24$) of Original Italian Grana Padano of different ages (6–22 months) were supplied by commercial dairy plants in Italy (Series I). Reference samples ($n = 10$) of Grana Padano (10–20 months), which had been certified by the Consorzio Grana Padano, were obtained from Prof. P. Resmini (Università degli Studi di Milano, I-20133 Milan) (Series II), additional reference samples ($n = 13$, 1–18 months) were obtained directly from the Consorzio Grana Padano (I-20121 Milan) (Series III).

Commercial Parmesan samples ($n = 49$ as a loaf or prepacked slices and $n = 68$ as grated products) were taken from retail outlets in Austria and Italy.

Parmesan cheese was produced in an Austrian cheese factory according to the traditional technology. The cheeses had a large cylindrical shape with a diameter of 40 cm and a height of 20 cm. At first, the manufacturing protocol for Parmesan cheese was followed exactly, but then two cheeses were removed from the ripening room at an age of 4 (Loaf A) and 2 (Loaf B) months. Cheese loaves were subsequently stored at lowered temperature (6 °C) for 18 months, so that their final age was 22 (Loaf A) and 20 months (Loaf B). At regular intervals, samples were taken using a cheese borer and kept frozen for further analyses.

2.2. Preparation of cheese proteins

After removal of the rind, cheese samples were grated, dried at 45 °C, defatted by extraction with petroleum ether in a Soxhlet apparatus and dried

at 30 °C. The resulting powder was dissolved in 10 mmol·L⁻¹ Tris-glycine buffer, pH 8.3, containing 6 mol·L⁻¹ urea and 3 % (v/v) 2-mercaptoethanol to give a final concentration of 1 % (w/v).

2.3. Urea-polyacrylamide gel electrophoresis (urea-PAGE)

Urea-PAGE analysis of caseins was performed by using a dual cooled vertical slab gel electrophoresis unit SE 600 (Hoefer Scientific Instruments, San Francisco, CA, USA). Separation gel (12 % T; 2.6 % C; 380 mmol·L⁻¹ Tris-citrate buffer, pH 8.9; 5 mol·L⁻¹ urea), stacking gel (5 % T; 7.5 % C; 120 mmol·L⁻¹ Tris-citrate buffer, pH 7.2; 5 mol·L⁻¹ urea) and electrode buffer (10 mol·L⁻¹ Tris-glycine buffer, pH 8.3) were prepared according to the procedure of Mayer [22], which was modified from Maurer [20]. Electrophoresis was carried out at 15 °C at a constant voltage of 200 V for 30 min and 400 V for 4 h. Gels were simultaneously fixed and stained with 0.15 % (w/v) Coomassie brilliant blue G-250 in water/methanol/acetic acid (60/40/10:v/v/v), destaining was done in water/methanol/acetic acid (70/30/7:v/v/v) [13]. All electrophoresis chemicals were of plusone™ quality (Pharmacia LKB Biotechnology, Uppsala, Sweden); other chemicals were of analytical grade (Merck, Darmstadt, Germany).

2.4. Densitometry

Densitometric evaluation of colour photos (Polaroid SX-70 instant film) of urea-PAGE gels was performed at 619 nm using Shimadzu (Kyoto, Japan) CS-910 densitometer and C-R1A integrator. Quantification was based on the measurement of peak areas of γ -casein (calculated as the sum of γ_2 , γ_3 and γ_1 -caseins [10]) and of β -casein. Reference samples of Grana Padano of different ages were used for calibration curves. Coefficients (γ -casein/ β -casein) were plotted against the age of cheese (months) and linear relationships were calculated for Series I and III. Determination of the β -casein content (g/100 g cheese solids non-fat) was performed by using β -casein (Sigma Chemical Co., St Louis, MO, USA) as an external standard.

2.5. HPLC analysis of free amino acids (FAA)

In Erlenmeyer flasks, 3 g of grated cheese were suspended in 27 g 100 mmol·L⁻¹ citrate-

HCl, pH 2.2 buffer. The mixture was homogenized with an Ultra Turrax (8 000 rpm for 2 min), moderately stirred for 20 min and filtered through a Schleicher & Schuell 595 1/2 folded filter. Five g 3 % (w/v) 5-sulfosalicylic acid were added to 1 g cheese filtrate and after 20 min of stirring, the suspension was filtered through a Schleicher & Schuell 595 1/2 folded filter. One g filtrate was diluted (1:50) with 50 mmol·L⁻¹ borate-HCl, pH 9.0 buffer and 20 µL samples were used for HPLC analysis of free amino acids. All chemicals were of analytical grade (Merck).

Free amino acid analysis was carried out by a high-performance liquid chromatography (HPLC) method according to the Waters AccQ.Tag® precolumn derivatization procedure using the Waters AccQ.Tag® chemistry package [30]. RP-HPLC was performed on a Waters Chromatography System (Waters Corp., Millford, MA, USA) using a model 600 E multisolvent delivery system, an U6K injector, guard column and an AccQ.Tag® amino acid analysis column (Nova-Pak™ C18, 4 µm). Column eluates were monitored at 395 nm (excitation at 250 nm) using a Shimadzu (Tokyo, Japan) RF-535 fluorescence detector interfaced with PC running Waters Maxima 820 software for automatic quantitation and documentation.

To prepare eluent A from Waters AccQ.Tag® Eluent A concentrate, 200 mL of the concentrate were added to 2 L Milli-Q™ water (Millipore, Bedford, MA, USA). Eluent B was HPLC-grade acetonitrile (Labscan Ltd, Dublin, Ireland), eluent C was Milli-Q™ water (Millipore). All eluents and derivatized samples were filtered through Millipore filters (HA-aqueous, 0.45 µm, FH-organic, 0.5 µm and GV, 0.22 µm). Samples were analysed in duplicate, injection volume was 5 µL. Elution was carried out at 37 °C following the instructions for single-pump gradient delivery systems [30]. The Waters amino acid hydrolysate standard solution, containing α -amino-n-butyric acid as an internal standard, was used for calibration in the range of 2.5 to 40 pmol. Concentrations of individual amino acids were displayed as pmol, which were subsequently converted to the total amount of amino acids calculated as g FAA per 100 g of cheese protein.

2.6. Kjeldahl determination of soluble nitrogen fractions

The following fractionation procedure was used only for Loaf A and Loaf B and was per-

formed according to the instructions reported previously [7, 8, 28] with modifications.

The total nitrogen (TN) content of the cheese samples was determined by the Kjeldahl method after mineralization [11].

Grated cheese (20 g) was suspended in 180 g deionized water at 50 °C. The mixture was homogenized with an Ultra Turrax apparatus (9 000 rpm for 2 min), moderately stirred for 1 h at 50 °C and subsequently homogenized as above. After centrifugation (10 000 g for 30 min at 5 °C) the suspension was filtered through a Schleicher & Schuell 595 1/2 folded filter. Cheese filtrate was finally filtered through a Millipore filter (GS, 0.22 µm), nitrogen soluble in water (WSN) was determined in duplicate using the Kjeldahl method.

Twenty mL of 24 % (w/v) trichloroacetic acid (TCA) solution was added to 20 mL of cheese filtrate. The suspension was held at room temperature overnight and then filtered through a Schleicher & Schuell 602 1/2 folded filter. The nitrogen content of the TCA-soluble fraction (TCA-N) was determined by the Kjeldahl method.

Twenty mL of 10 % (w/v) phosphotungstic acid (PTA) solution was added to 20 mL of cheese filtrate and held at room temperature overnight. The nitrogen content of the PTA-soluble fraction (PTA-N) was determined after filtration using the Kjeldahl method. All chemicals were of analytical grade (Merck).

Nitrogen fractions soluble in water (WSN), 12 % TCA (TCA-N) and 5 % PTA (PTA-N) were calculated as relative content of the total nitrogen (% of TN).

3. RESULTS

3.1. Urea-PAGE casein patterns and densitometry

Figure 1 shows the electrophoretic patterns obtained by urea-PAGE analysis of the reference samples of Grana Padano of Series I. As a result of the action of plasmin, which seems to be one of the most active endopeptidases in Grana Padano, β -casein is hydrolysed rapidly during the first year of ripening and the γ -caseins are accumulated as its major degradation products. These peptides are present even in

4 years old Parmigiano-Reggiano cheese (figure 1, right lane). Since the true composition of the γ -casein fraction depends on the genetic variants of β -casein occurring in the bulk milk used for cheesemaking [10], this fraction was summarized as ' γ -casein' for simplification. α_{S1} -casein is the principal target for proteolysis during the early stages of ripening. Consequently, α_{S1} -I-casein, as its primary degradation product, continues to

increase until the 12th month of ripening, and is subsequently hydrolysed to yield α_{S1} -II-casein and other peptides. As the differentiation and identification of these degradation products was not an object of this study, this fraction was termed ' α_{S1} -II-casein' [16].

Referring to the breakdown of β -casein, the ripening characteristics of Parmesan cheese becomes even more evident when

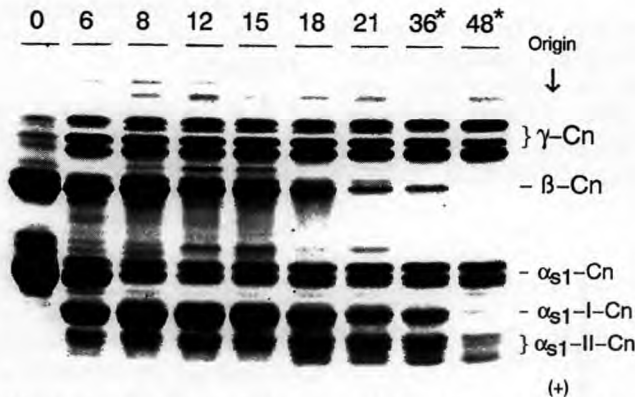


Figure 1. Urea-PAGE analysis of reference samples of Grana Padano (Series I) and of two additional samples of Parmigiano-Reggiano (indicated by an asterisk). Numbers refer to the age of cheese (months).

Figure 1. Électrophorégramme urée-PAGE des échantillons de référence de Grana Padano (série I) et de deux échantillons de Parmigiano-Reggiano (indiqués par un astérisque). Les numéros correspondent à l'âge du fromage (en mois).

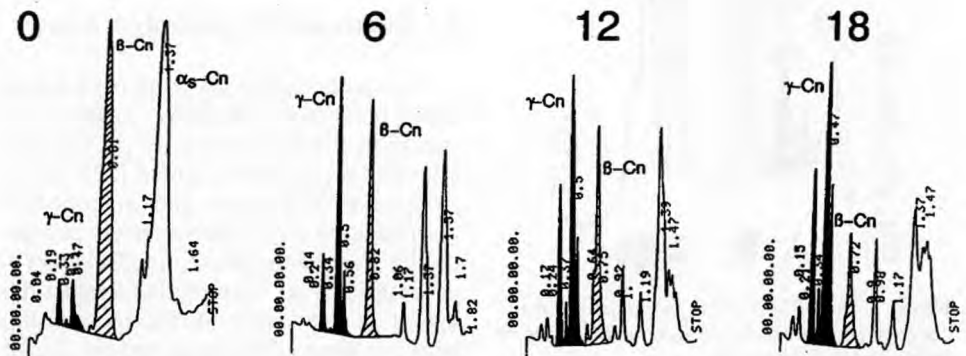


Figure 2. Densitometric curves of selected reference samples of Grana Padano (Series I). Numbers refer to the age of cheese (months).

Figure 2. Courbes densitométriques de quelques échantillons de référence de Grana Padano (série I). Les numéros correspondent à l'âge du fromage (en mois).

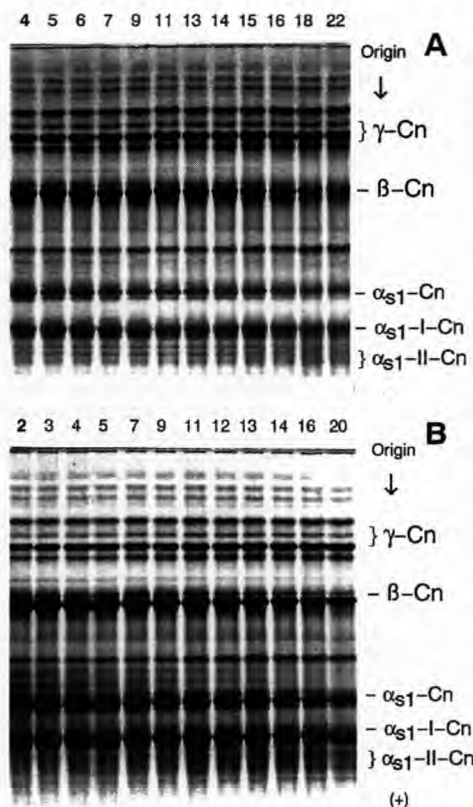


Figure 4. Urea-PAGE analysis of Parmesan samples. **A**, Loaf A; **B**, Loaf B. Numbers refer to the age of cheese (months).

Figure 4. Électrophorégramme urée-PAGE d'échantillons de Parmesan. **A**, meule A; **B**, meule B. Les numéros correspondent à l'âge du fromage (en mois).

sed. Based on this calibration a standard level ($\gamma\text{-Cn}/\beta\text{-Cn}$) of 1.512 ($\sigma = 0.106$) was found for 12-month-old Grana Padano cheese. Taking into account a 2 σ confidence level we propose to use a threshold level of 1.3 ($\gamma\text{-Cn}/\beta\text{-Cn}$) to verify the required age of 12 months for all Parmesan-type cheeses retailed as a loaf or prepacked slices, since β -casein breakdown is even more extensive in Parmigiano-Reggiano in comparison to Grana Padano [1]. Although coefficient $\gamma\text{-Cn}/\beta\text{-Cn}$ shows a high degree of variability, most samples of Series II sho-

wed an even higher extent of proteolysis in comparison to samples of Series I and all samples except one had a coefficient ≥ 2.0 (one 10-month-old sample had a coefficient of 1.6). This means that all samples of Series II more than fulfilled the requirements of the proposed threshold level of 1.3 ($\gamma\text{-Cn}/\beta\text{-Cn}$). Samples of Series III do not show any appreciable increase referring to this proteolysis index, due to the reasons described above.

At first, in particular Loaf A shows even higher coefficients ($\gamma\text{-Cn}/\beta\text{-Cn}$) in comparison to that observed in the reference samples of Series I. However, during further storage at lowered temperature, coefficients increase very slowly. Because of its very high initial coefficient, Loaf A exceeds the proposed threshold level of 1.3 at an age of 14 months, although the standard level of 1.5 is not reached before an age of 18 months. Loaf B does not exceed these levels during the storage period.

As the minimum age for grated Grana Padano was established to be 9 months [12], the corresponding standard level for grated Parmesan cheese is 1.0 ($\gamma\text{-Cn}/\beta\text{-Cn}$). Since commercial grated Parmesan samples may contain $\leq 20\%$ cheese rind [12, 26], which has a very low extent of proteolysis [22, 24], a threshold level of 0.8 ($\gamma\text{-Cn}/\beta\text{-Cn}$) is proposed for all grated Parmesan products. This value corresponds to the proteolysis index of an approximately 6-month-old loaf of Grana Padano (Series I).

Equivalent results were obtained with the β -casein content of the reference samples (figure 6). β -casein decreases rapidly in samples of Series I and II. The relationship between β -casein content and the age of reference samples of Series I was linear in the range analysed. In most cases, samples of Series II showed an even more extensive breakdown of β -casein. In striking contrast to that, even very old samples of Series III had a higher β -casein content than every other reference sample analysed in this study. Thus the question arises whether these

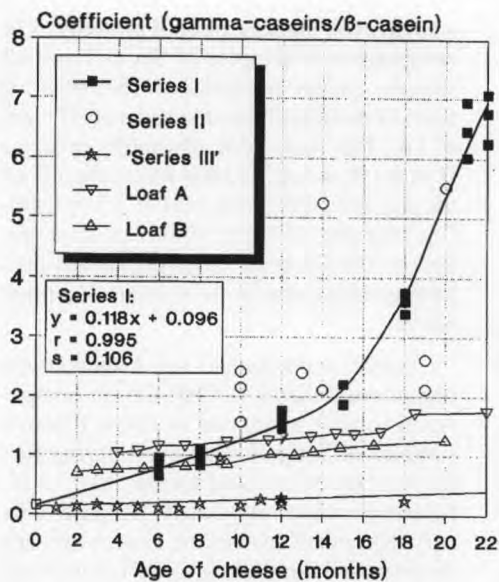


Figure 5. Comparison of three series of reference samples of Grana Padano as well as of Loaf A and B referring to the coefficient (γ -Cn/ β -Cn). Linear regression up to 15 months of ripening is shown for Series I.

Figure 5. Comparaison des trois séries d'échantillons de référence de Grana Padano ainsi que des meules A et B pour le coefficient (γ -Cn/ β -Cn). La régression linéaire jusqu'à 15 mois est montrée pour la série I.

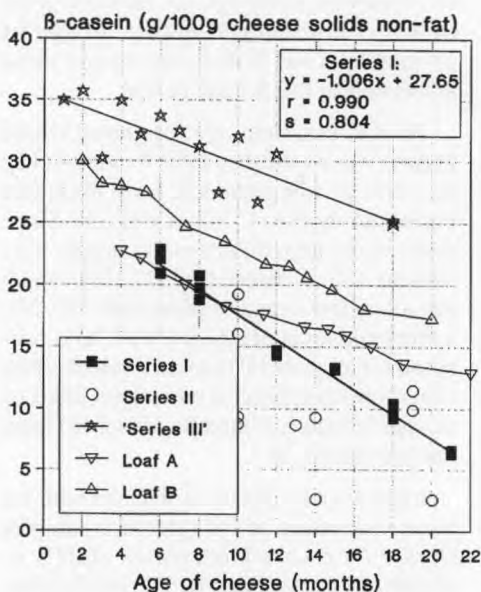


Figure 6. Comparison of three series of reference samples of Grana Padano as well as of Loaf A and B referring to the β-casein content. Linear regression is shown for Series I.

Figure 6. Comparaison des trois séries d'échantillons de référence de Grana Padano et des meules A et B pour la teneur en caséine β . La régression linéaire est montrée pour la série I.

cheese samples were in fact Grana Padano cheese produced following the traditional technology of manufacture. There is no reasonable explanation for the phenomenon that very little endoprotease activity and, in particular, nearly no β -casein breakdown occurs in Grana Padano cheese samples of

Series III, unless these cheeses were not ripened using traditional ripening conditions, but were stored at lowered temperature or ripened following a new technology.

At first, in particular, Loaf A has a very low β -casein content in comparison to that observed in reference samples of Series I.

However, during further storage at 6 °C, β -casein content decreases at a reduced rate. In consequence, β -casein content of loaves (in particular, Loaf B) is relatively high at an advanced age in comparison to that of reference samples of Series I. Loaf A exceeds the standard level of approximately 15 g β -casein per 100 g cheese non-fat solids, which corresponds to a 12-month-old reference sample of Series I, only at an age of 18 months, whereas Loaf B does not reach this level during the storage period.

3.3. HPLC analysis of free amino acids

Figure 7 shows the total amount of free amino acids (FAA) plotted against the age of Parmesan cheese. Although FAA content of reference samples of Series I and II shows a very high degree of variability, a distinct increase of FAA content can be observed during the ripening period. Referring to the analysis of FAA, reference samples of Series III show a very high extent of proteolysis, which is in striking contrast to the results obtained by urea-PAGE analysis of caseins.

If we consider the fact that reference samples of Series I and III have completely different electrophoretic patterns (figures 1, 2 and 3), it is quite surprising that in almost all cases, samples of Series III show a higher FAA content. In consequence of these findings, reference samples of Series I and III cannot be distinguished using HPLC analysis of total FAA, although there is a highly significant difference in the coefficient (γ -Cn/ β -Cn) (figure 5) and the β -casein content (figure 6).

FAA content of Loaf A and B shows a steady increase during the storage at 6 °C. Taking into account the fact that Loaf A and B had a very limited ripening period of only 4 and 2 months, respectively, it is quite surprising that at an advanced age, their FAA contents stay within the variability of those of reference samples of Series I and II.

3.4. Soluble nitrogen fractions

Figure 8 shows the nitrogen fractions soluble in water (WSN), 12 % TCA (TCA-N) and 5 % PTA (PTA-N) calculated as

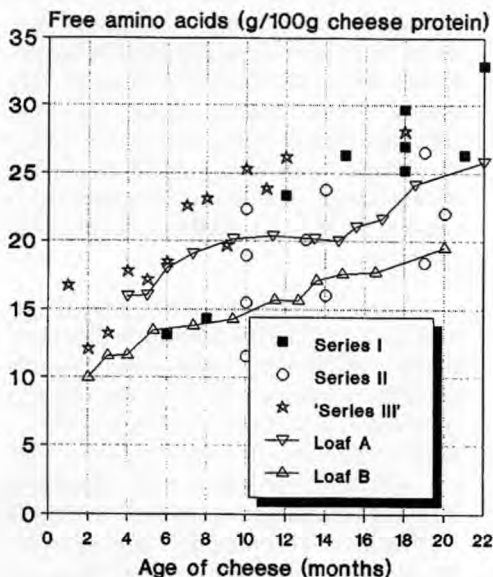


Figure 7. Comparison of three series of reference samples of Grana Padano as well as of Loaf A and B referring to the FAA content.

Figure 7. Comparaison des trois séries d'échantillons de référence de Grana Padano, ainsi que des meules A et B pour la teneur en acides aminés libres.

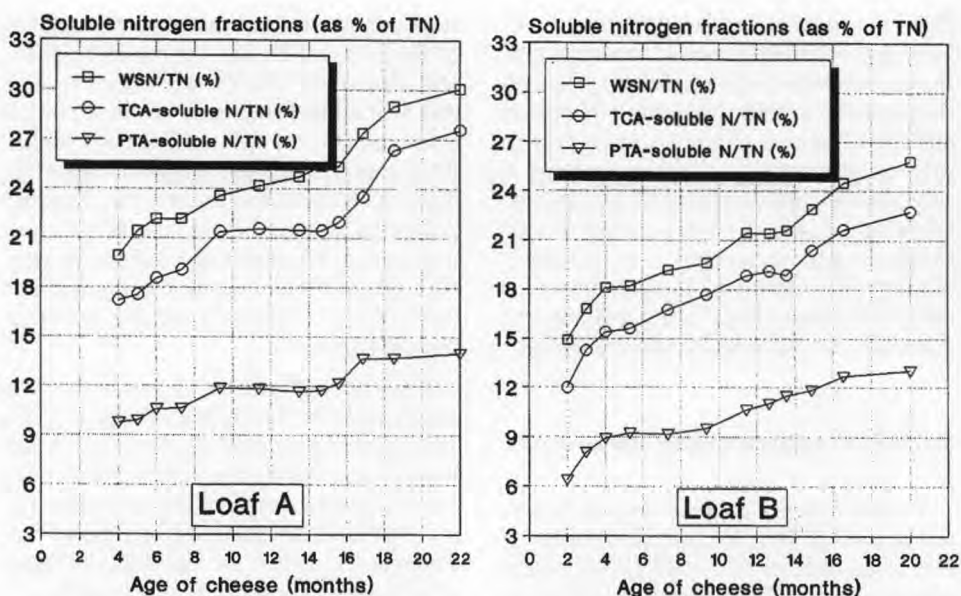


Figure 8. Changes of soluble nitrogen fractions as relative content of the total nitrogen (TN) during the storage period of Loaf A and B at 6 °C.

Figure 8. Évolution des fractions solubles de la matière azotée exprimée par rapport à la teneur totale en matière azotée (TN) au cours du stockage à 6 °C des meules A et B.

relative content of the total nitrogen (% of TN). Soluble nitrogen fractions of Loaf A and B show a steady increase during the storage period at 6 °C. Because of its high initial level of WSN, Loaf A reaches a very high final WSN content of 30 %. Loaf B starts at a relatively low WSN level, but reaches 26 % WSN at the end of the storage period.

3.5. Commercial Parmesan samples

Commercial Parmesan samples labelled as Grana Padano, Parmigiano-Reggiano or Original Italian Parmesan cheese were taken from retail outlets in Austria and Italy. Figure 9 shows the electrophoretic casein patterns of selected commercial Parmesan samples retailed as a loaf or as grated products. Most of the samples, retailed as a loaf or as prepacked slices, show electrophoretic patterns that are in good accordance with

those obtained by the reference samples of Series I and II, having weak β -casein bands and very intense bands in the γ -casein region. However, many of the grated products show quite surprising electrophoretograms, which are similar to those of very young Grana Padano cheese or whole reference casein. Using urea-PAGE analysis, intense bands of α_{S1} - and β -casein, but no degradation products could be detected in many of these commercial samples of grated Parmesan.

Figure 10 illustrates the relative distributions of the samples referring to their coefficients (γ -Cn/ β -Cn) as well as their β -casein contents. Less than 10 % of the samples (retailed as a loaf or as prepacked slices) have coefficients (γ -Cn/ β -Cn) lower than 1.3, which was proposed as the threshold level for verification of the required age of 12 months. Concomitantly, only approximately 10 % of the samples have a β -casein

content higher than 15 g per 100 g cheese non-fat solids. This standard level corresponds to the degree of β -casein degradation of a 12-month-old reference sample of Grana Padano (Series I).

In striking contrast to these findings, more than 60 % of the commercial grated Parmesan samples have coefficients (γ -Cn/ β -Cn) lower than 0.8, which was proposed as the threshold level for verification of the required age of 9 months. Concomitantly, approximately 60 % of the samples have a β -casein content higher than 20 g per 100 g cheese non-fat solids, which corresponds to the extent of β -casein breakdown of a 8-month-old reference sample of Grana Padano (Series I).

4. DISCUSSION

Electrophoretic patterns obtained by urea-PAGE analysis of the reference samples of Grana Padano of Series I and II were in good

accordance with those reported in other studies [1, 6, 15, 22, 23]. α_{S1} -casein is the principal target for proteolysis during the early stages of ripening, which was also observed in other cheese varieties [21]. As a result of the action of plasmin, which seems to be one of the most active endopeptidases in Grana Padano, β -casein is hydrolysed rapidly during the ripening period and the γ -caseins are accumulated as its major degradation products. As the sum of all γ -caseins was used for the calculation of the coefficient (γ -Cn/ β -Cn), we did not differentiate between the distinct γ -caseins (γ_1 -, γ_2 - and γ_3 -caseins deriving from the different genetic variants of β -casein), which can only be separated by isoelectric focusing. As we could find a residual β -casein band, even in 22-month-old Grana Padano, we doubted this fraction to be really β -casein, because, using two-dimensional electrophoresis, this band proved to contain several degradation products of β -casein, which have the same electrophoretic mobility in one-dimensional urea-PAGE [1].

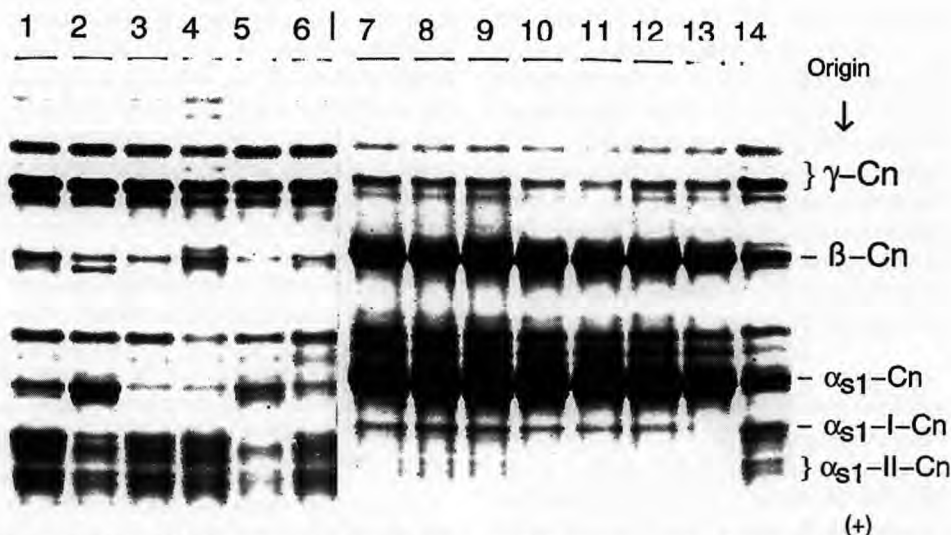


Figure 9. Urea-PAGE analysis of selected commercial Parmesan samples. Lanes 1–6, retailed as a loaf; 7–14, as grated product.

Figure 9. Électrophorégramme urée-PAGE de quelques échantillons de Parmesan du commerce. Lignes 1–6, vendu à la coupe ; 7–14 vendu en râpé.

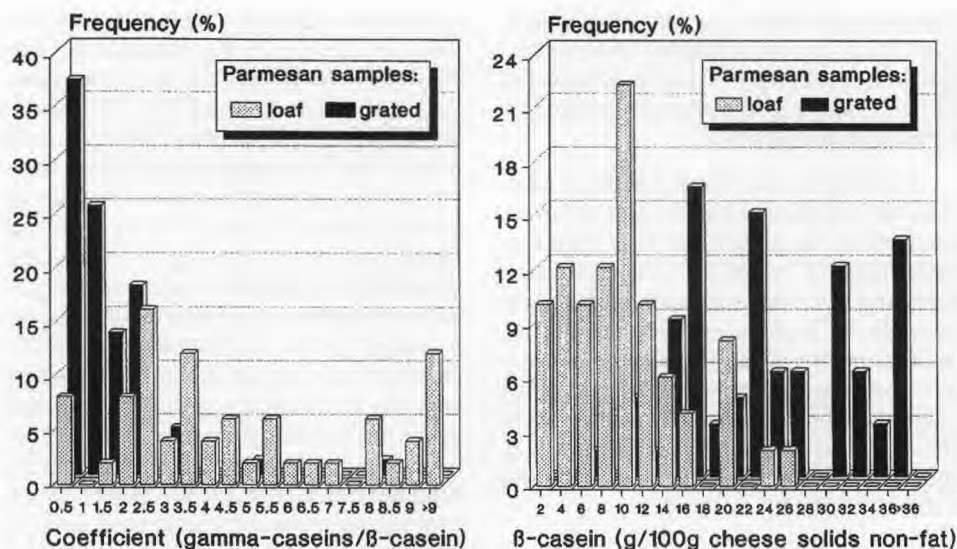


Figure 10. Relative distributions of commercial Parmesan samples retailed as a loaf or prepacked slices ($n = 49$) or as grated product ($n = 68$) referring to the coefficient (γ -Cn/ β -Cn) and β -casein content.

Figure 10. Distribution relative des échantillons de parmesan du commerce vendu à la coupe, en préemballé ($n = 49$), ou en râpé ($n = 48$), selon le coefficient (γ -Cn/ β -Cn) et la teneur en caséine β .

Threshold levels (γ -Cn/ β -Cn) were proposed for the verification of the required age of Parmesan cheese retailed as a loaf or as grated product. The use of these threshold levels as a standard to assess the endoproteolytic changes of commercial samples could contribute to improve the quality of Parmesan cheese. In comparison to Grana Padano samples of Series I and II, very similar electrophoretograms were also reported for a Parmesan-type cheese manufactured in Austria [22].

Commercial Parmesan samples retailed as a loaf or as prepacked slices were generally found to fulfill the requirements concerning endoproteolytic changes during ripening. However, many grated Parmesan samples taken from retail outlets in Austria showed poor quality, which was probably due to the adulteration with products with low proteolysis (e.g., cheese rind, very young cheese).

Reference samples of Grana Padano of Series III showed quite surprising electrophoretic patterns of caseins, which were similar to those of very young Grana Padano cheese. Neither the breakdown of β -casein, nor a distinct degradation of α_{S1} -casein, could be observed during the ripening period of 18 months. Based on the electrophoretic study of caseins, in all cases the degree of proteolysis was not related to the labelled age of these samples, which had been certified by the Consorzio Grana Padano. However, using HPLC analysis of FAA, samples of Series III showed a very high extent of proteolysis, which was in striking contrast to the results obtained by urea-PAGE analysis of caseins.

As an experiment, two loaves of Parmesan cheese were removed from the ripening room at an age of 4 and 2 months and were subsequently stored at 6 °C for 18 months. During the storage period, endoproteolytic changes were slowed down and breakdown

of α_{S1} - and β -casein as well as accumulation of degradation products proceeded at reduced rate, which could be detected using urea-PAGE casein patterns, but not by HPLC analysis of FAA content. In comparison to the results reported in other studies [5, 27], these loaves could not be distinguished from Grana Padano cheese produced according to the traditional technology using HPLC analysis of their FAA content. At an age of 12 months, Loaf A and B have a FAA content of approximately 20 and 16 g per 100 g of cheese protein, respectively, which is also within the variability of the FAA content of 12-month-old reference samples of Series I and II.

At an age of 12 and 22 months, Loaf A reaches a WSN content (as % of TN) of approximately 25 % and 30 %, respectively, which is also within the variability reported for reference samples of Grana Padano of the same age [5, 6].

In conclusion, since Kjeldahl determination of WSN as well as HPLC analysis of FAA content give insufficient information on the distinct endoproteolytic changes, which were found to be typical for Parmesan cheese, urea-PAGE of the casein fraction has to be done to enable the evaluation of the extent of proteolysis and therefore a deduction of the age of commercial Parmesan samples.

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