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Original article

Analysis of bovine caseins and primary hydrolysis products in cheese by capillary zone electrophoresis

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Summary — Casein fractions from milk and various cheeses as well as isolated casein standards were analysed by capillary electrophoresis under acidic conditions. Capillary electrophoresis was performed on a Waters Quanta 4000 apparatus with a hydrophilically coated or an untreated fused-silica capillary. The four major caseins in an acid precipitate from milk were well separated. In addition, the various phosphorylation states of the α_{s1}- and the α_{s2}-caseins and some genetic variants of β-casein were separated. The major casein hydrolysis products formed in cheese, para-κ-casein, α_{s1}-casein–I and γ-caseins, were separated from their parent casein and from most other major caseins. The linearity of the method with respect to injection time and casein concentration was acceptable. With improved repeatability of peak areas the method should be superior to urea–PAGE and an attractive alternative to high-pressure liquid chromatography (HPLC) methods for following casein proteolysis in dairy products.

capillary electrophoresis / bovine casein / cheese

Résumé — Analyse des caséines bovines et des produits d'hydrolyse primaire du fromage par électrophorèse capillaire. Des fractions de caséine du lait et de divers fromages, ainsi que des standards de caséines isolées ont été analysés par électrophorèse capillaire en milieu acide. L'électrophorèse capillaire (EC) a été réalisée avec l'appareil Quanta 4000 de Waters, avec des capillaires traités par un polymère hydrophile et des capillaires en silice non traités. Les quatre caséines majeures d'un précipité acide du lait ont bien été séparées par l'EC. De plus, les caséines α_{s1} et α_{s2} avec un nombre de groupes phosphate variable et quelques variants génétiques de caséine β ont été séparés. Leurs produits de dégradation principaux dans le fromage, caséines para-κ, α_{s1}-I et γ, ont été séparés de leur caséine originale et de la plupart des autres caséines. La linéarité de la méthode concernant la durée d'injection et la concentration des caséines étaient suffisantes. Avec une amélioration de la répétabilité des aires de pic, cette méthode serait meilleure que l'électrophorèse en gel de polyacrylamide en présence d'urée et représenterait une alternative intéressante aux méthodes de chromatographie liquide haute performance pour évaluer la protéolyse primaire des caséines des produits laitiers.

électrophorèse capillaire / caséine bovine / fromage

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INTRODUCTION

Bovine milk contains four different caseins, α_{s1}-, α_{s2}-, β- and κ-casein, three of them occurring as several genetic variants. The α_s-caseins, in addition, contain a varying number of serine phosphate groups (Swaisgood, 1992). During manufacture and ripening of cheese the caseins are broken down to various degrees, the extent of which is a major determinant of the texture and flavour of the cheese (Creamer and Olson, 1982; Fox et al, 1993). Cleavage of κ-casein catalysed by chymosin (EC. 3.4.23.4) leads to milk coagulation and formation of the cheese curd. Primary proteolysis of the caseins in the cheese is initiated by the action of the added chymosin on α_{s1}-casein and of the indigenous plasmin (EC. 3.4.21.7) on β-casein (Grappin et al, 1985; Exterkate and Alting, 1995). Further proteolysis during cheese ripening is catalysed mainly by proteases and peptidases released from starter and other bacteria (Fox et al, 1993). The main primary casein hydrolysis products reported to occur in cheese are listed in table I.

To characterise the casein proteolysis during cheese maturation, the pH 4.6-insoluble protein fraction of cheeses have been analysed by various methods (Grappin and Ribadeau-Dumas, 1992). Traditional slab gel electrophoresis in urea containing media has the potential for resolution of all four major caseins as well as some of the α_{s2}-casein forms and a range of casein hydrolysis products (Creamer, 1991; van Hekken and Thompson, 1992; Centeno et al, 1994; McSweeney et al, 1995). Due to the laborious and inaccurate quantitation using this technique, chromatographic techniques have been developed for analysis of caseins (Andrews et al, 1985; Christensen et al, 1989; Hollar *et* al, 1991; Strange et al, 1991; Syväoja, 1992; Calvo et al, 1992; Ng-Kwai Hang and Chin, 1994). Although some of these can separate the major caseins in a whole casein fraction (Hollar et al, 1991), in most chromatograms α_{s1}-casein and α_{s2}-casein are not baseline-separated, and the picture can be complicated when casein fractions from cheeses are run (Christensen et al, 1989; Exterkate and Alting, 1995).

Capillary electrophoresis (CE), a technique successfully used for analysis of a range of proteins (Grossman et al, 1989; Lindner et al, 1993; Werner et al, 1993; Otte et al, 1994), is an alternative to traditional slab gel electrophoresis and is complementary to liquid chromatography. In CE, separation is based on charge to mass ratio differences of the components as in gel

Table I. Major casein hydrolysis products found in cheese (Fox et al, 1993).
Principaux produits d'hydrolyse de la caséine retrouvés dans le fromage.

Casein	*Fragment*	*Trivial name*
κ-Casein (chymosin)	1 → 105	*para*-κ-casein
α_{s1}-Casein (chymosin)	24/25 → 199	α_{s1}-Casein-I
β-Casein (plasmin)	29 → 209	γ_1-Casein
	106 → 209	γ_2-Casein
	108 → 209	γ_3-Casein

electrophoresis, but separation and quantification can be performed in one step as in high-pressure liquid chromatography (HPLC). Furthermore, the resolution potential of CE is higher than for HPLC (Grossman et al, 1989), only small volumes of buffer and sample are needed, generally non-toxic buffers are used, and capillaries used for CE are much cheaper than HPLC columns.

A few studies concerning CE analysis of caseins have been performed (Chen and Zhang 1992; de Jong et al, 1993; Kanning et al, 1993; Kristiansen et al, 1994). Kristiansen et al (1994) have shown that the four major caseins occurring in acid casein were separated at neutral pH in an untreated capillary. The separation could be improved by increasing the pH and using a buffer modifier and a coated capillary, and casein separation patterns resembling urea–PAGE patterns were obtained (unpubl results). However, the whey proteins were not separated from the caseins, which can present a problem if cheeses containing denatured whey proteins are analysed, or if the caseins are not isoelectrically separated from any native whey proteins present before analysis. De Jong et al (1993) have shown that a good analytical separation of all major milk proteins can be obtained by CE at low pH in coated capillaries using a polymeric buffer additive. The migration behaviour of the most phosphorylated forms of the α_{s1}- and α_{s2}-caseins, however, was not investigated. Furthermore, no information on the migration of the major casein fragments produced during manufacture and ripening of cheeses is available.

The purpose of the present study was to apply the method of de Jong et al (1993) with slight modifications for analysis of caseins from cheese, and to identify the major caseins and primary casein hydrolysis products in cheese, and discuss the suitability of the method for determination of casein proteolysis occurring in cheeses.

MATERIALS AND METHODS

Materials

Trisodium citrate dihydrate, ammonium acetate, sodium dihydrogen phosphate monohydrate and urea were of analytical grade and obtained from Merck (Merck–Darmstadt, Germany). Hydroxypropyl methyl cellulose (HPMC, no 20.032-8, 2% solution = 4000 cps) was from Aldrich-Chimie (Steinheim, Germany). All solutions were based on highly purified water (Milli-Q Plus, Millipore Corp, Bedford, MA, USA).

Fresh milk from two cows, one homozygotic for α_{s1}-casein B, β-casein A1 and κ-casein A, the other homozygotic for α_{s1}-casein B, β-casein A2 and κ-casein B, was obtained from the National Institute of Animal Science (Research Centre Foulum, Denmark). Acid casein was prepared from the milk as described by Kristiansen et al (1994). Fractions of pure α_{s1}-casein and β-casein were obtained by ion exchange of the acid casein containing β-casein A1 (Kristiansen et al, 1994). Pure casein standards of α_{s2}-, β- and κ-casein, isolated as described by Rasmussen et al (1992a), were obtained from the Protein Chemistry Laboratory (University of Aarhus, Denmark). Other samples of β-casein (C-6905) and κ-casein (C-0406) as well as the tripeptide Lys-Tyr-Lys (L-3271) were obtained from Sigma Chemical Co (St Louis, MO, USA). Rennet whey was prepared from fresh bulk milk as described by Otte et al (1994). Caseinomacropeptide was a gift from MD Foods Ingredients (Videbaek, Denmark).

Bovine chymosin (Chymogen, type B, 44 CHU/mL) was from Christian Hansen A/S (Hørsholm, Denmark). Bovine plasmin (no 602 370, 5 U/mL) was from Boehringer–Mannheim GmbH (Mannheim, Germany).

Mozzarella (one to five weeks old) and Feta (six and 40 weeks old) cheeses were manufactured from ultrafiltered (UF) bovine milk for other projects at the department. The Danbo cheese (12 weeks old) was supplied by Chr Hansen A/S.

Extraction of cheese

Ten g of grated cheese was dissolved in 40 mL 0.5 mol/L sodium citrate buffer, pH 8.5, at 40 °C with magnetic stirring for ~ 1 h. The volume was

made up to 200 mL with distilled water, and the suspension was centrifuged (4 °C, 20 min, 2500 *g*) and/or filtered through glass wool. The pH of the supernatant was lowered to 4.6 with 1 mol/L HCl, and after centrifugation the supernatant was discarded and the precipitate allowed to drain. The drained precipitate was dissolved in sample buffer (see below). In a few instances the filtered citrate suspension of caseins was simply diluted with an equal volume of sample buffer.

Hydrolysis of α_s- and κ-casein with chymosin

α_s-Casein and κ-casein were dissolved at 10 mg/mL in 0.075 mol/L ammonium acetate buffer, pH 6.2, and hydrolysed with 0.3 and 0.003% chymosin respectively, as described by Kristiansen et al (1994). Samples (100 µL) withdrawn at different intervals after enzyme addition (α_s-casein: t = 0, 1/2, 1, 2 and 4 h; κ-casein: t = 0, 5, 10, 20 and 50 min) were immediately boiled for 5 min and cooled to room temperature. The pH was lowered to 4.6, and after equilibration for 20 min, the precipitates were collected by centrifugation (11 000 *g*, 5 min).

Hydrolysis of β-casein with plasmin

Two samples of β-casein (10 mg/mL), one containing only β-casein A1 and one containing a mixture of genetic variants (from Sigma), were hydrolysed with plasmin (10 µL/850 µL of sample) in 0.05 mol/L phosphate buffer, pH 7.0, at 37 °C. The caseins in the 100-µL samples withdrawn after 1, 2, 3, 4 and 23 h of incubation with plasmin were precipitated at pH 4.6, centrifuged, and evaporated to dryness (Hetovac VR-1, Heto Lab Equipment A/S, Allerød, Denmark).

Sample pretreatment

To dissociate the caseins and insoluble hydrolysis products, all samples were dissolved in a sample buffer containing 8 mol/L urea and 10 mmol/L dithioerythritol (DTE) at pH 8, and left for at least 1 h at room temperature before filtration (0.45 µm Minisart, Sartorius) and CE analysis. The isoelectrically precipitated casein was dissolved at 30 mg/mL and the purified casein standards at 10 mg/mL. Casein precipitate from 100 µL hydrolysate was dissolved in 80 µL sample buffer (~ 13 mg/mL) and casein precipitate corresponding to approximately 0.1 g (Feta) or 1 g of cheese (UF-Mozzarella and Danbo) was dissolved in 2 mL sample buffer; the samples from UF–Mozzarella and Danbo cheeses were further diluted two and three times respectively with sample buffer before analysis. To most samples, additionally 1 µL of the tripeptide Lys-Tyr-Lys (50 mg/mL) was added per 50 µL of sample as a reference compound.

Capillary electrophoresis

Analysis was performed on a Waters Quanta 4000 Capillary Electrophoresis System, with 50 µm-id capillaries, either hydrophilically coated (CElect™-P150, Supelco, Inc, Bellafonte, PA, USA) or untreated (J&W Scientific, Folsom, CA, USA). Unless otherwise stated, the total length of the coated capillary was 43 cm and the untreated capillary 60 cm. The sample was injected hydrodynamically at the cathodic end for 15–30 s. The run buffer was 10 mmol/L sodium phosphate with 6 mol/L urea, pH 2.5, made up as described by de Jong et al (1993), but with 0.05% (or 0.02%) (w/v) hydroxypropyl methyl cellulose (HPMC) instead of methylhydroxyethylcellulose (MHEC). Separation was performed at constant voltage, resulting in a current of approximately 40 µA. The components were detected on column 7.5 cm from the anode by their UV absorbance at 214 nm. Millennium™ 2010 Chromatography Manager version 2.1 (Waters Chromatography Div, Milford, MA, USA) was used for data collection and processing. Between runs the capillary was purged for 3 or 7 min with run buffer. The first electrophoregram in a series was always discarded.

The linearity of the method in the untreated capillary was assessed with a sample from UF-Feta by varying the sample injection time from 5 to 40 s, followed by variation from 40 to 5 s. Peak areas were calculated as the average from the two determinations. Standard curves were made with varying concentrations (2 to 20 mg/mL sample buffer) of the purified α_s-casein and β-casein A1 fractions, respectively; each concentration was analysed only once.

The repeatability using the untreated and the coated capillary respectively was assessed by nine consecutive injections of acid casein and

caseins from UF-Feta cheese, respectively. All injections in one series were performed without exchange of buffer.

RESULTS AND DISCUSSION

Separation of caseins

By use of HPMC in the CE run buffer, the casein separation pattern obtained with the hydrophilically coated capillary (fig 1, top) was very similar to that obtained when MHEC was used (de Jong et al, 1993). Comparison with the results of de Jong et al (1993) suggests that the peak migrating at 20 min in figure 1 is α_{s2}-casein and that the peaks migrating at ~22 min and ~25 min are α_{s1}- and β-casein, respectively. The peak at ~24.5 min similarly should be κ-casein. These designations were confirmed by analysis of individual casein standards, as shown in the lower part of figure 1.

Despite minor day-to-day variations in migration times (t_m), the peaks for the caseins in the standards can be easily recognized in the electrophoregram of the acid casein (fig 1). It can be seen that α_{s2}-casein is separated into at least four peaks migrating between 19 and 21 min, which is consistent with the existence of four phosphorylation states of this protein containing ten to 13 phosphate groups (Swaisgood, 1992). The additional peaks might stem from disulphide-linked dimers from variants with different numbers of phosphate groups (Hoagland et al, 1971; Rasmussen et al, 1992b), as these dimers are only partly reduced by addition of DTE (Rasmussen and Petersen, 1991).

Apart from α_{s1}-casein (t_m ~21.5 min), the sample used as standard for α_{s1}-casein apparently also contained α_{s2}-casein and a few other components. The distance between the α_{s1}-casein peak and the following peak (t_m ~23 min) is similar to the distance between the two highest α_{s2}-casein peaks, that differ by only one phosphate

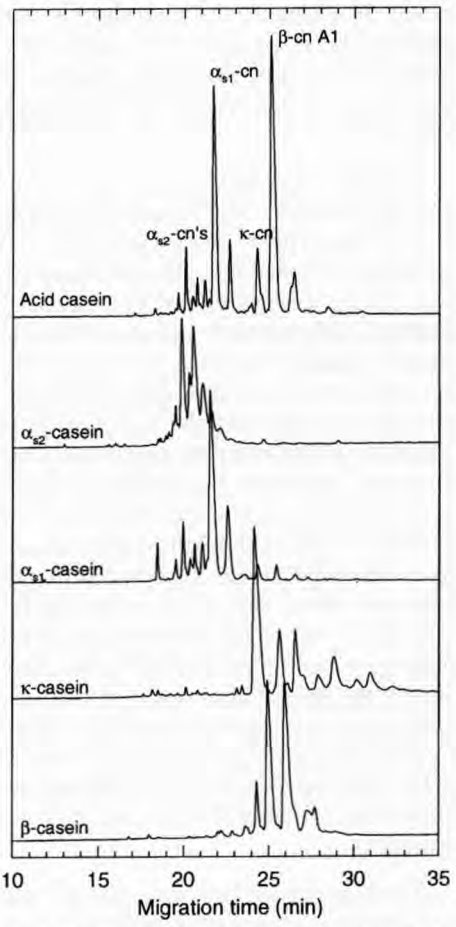

Fig 1. Capillary electrophoretic separation of the caseins in acid casein containing β-casein (β-cn) A1 (upper electropherogram) and identification of major caseins by analysis of casein standards. The scale of the acid casein is enlarged by a factor of 1.7 in comparison to the standards. Separation was performed in a coated capillary at 14 kV (~ 40 μA). Other conditions are described in the Methods section.

Séparation par électrophorèse capillaire des caséines d'un précipité acide de caséine contenant la caséine β (β-cn) A1 (électrophérogramme en haut) et identification des caséines principales par analyse des standards. L'échelle de la caséine acide est aggrandie d'un facteur de 1,7 par rapport aux standards. La séparation est réalisée avec un capillaire traité à 14 kV (~ 40 μA). Les autres conditions sont décrites dans la section 'Méthodes'.

group. The peak at ~23 min, thus, is supposed to be α_{s0}-casein (α_{s1}-casein 9P), which contains one phosphate group more than α_{s1}-casein (α_{s1}-casein 8P; Swaisgood, 1992).

κ-Casein separated into a range of components of which ~ 50% migrated as a peak at ~24.5 min (fig 1). κ-Casein is prepared from bulk milk and thus should contain the two genetic variants, A and B, in approximately equal amounts (Swaisgood, 1992). In figure 1, however, only one dominating peak is visible, which is in accordance with complete protonisation at pH 2.5 of Asp148 in variant A, and thus similar net charge as the B variant (Ala148). The following peaks are not supposed to be multimers of κ-casein, as these should be fully reduced by 9 mmol/L DTE (Rasmussen and Petersen, 1991). They might represent glycoforms of κ-casein containing *N*-acetyl neuraminic acid, as Otte et al (1995) have shown, that the C-terminal part of κ-casein, containing this negatively charged carbohydrate, has a longer migration time at pH 2.5 than the C-terminal part without carbohydrates or with neutral carbohydrate moieties.

The β-casein standard (fig 1, bottom) also was prepared from bulk milk and thus should contain several genetic variants dominated by A1 and A2. Accordingly, the migration of the first major β-casein peak at 25 min coincides with the β-casein peak in acid casein containing only β-casein A1 (fig 1, top). Confirmation of the β-caseins being A1 and A2, respectively (in that order) is given below (fig 2). From their primary structure, the minor β-casein genetic variants B and C are expected to migrate ahead of the A1 variant, and A3 to migrate behind the A2 variant, respectively. It is thus possible that two of the minor peaks in front of β-casein A1 represent the B and C variants of β-casein, and that one of the components migrating behind β-casein A2 represents the A3 variant. These minor peaks were not identified.

Figure 2 shows that the predominant β-casein variants, A1 and A2, are well separated by the method used, and that A1 migrates ahead of A2. The A1 variant of β-casein contains His (instead of Pro) at position 67, which is positively charged below pH 6 and results in a higher mobility

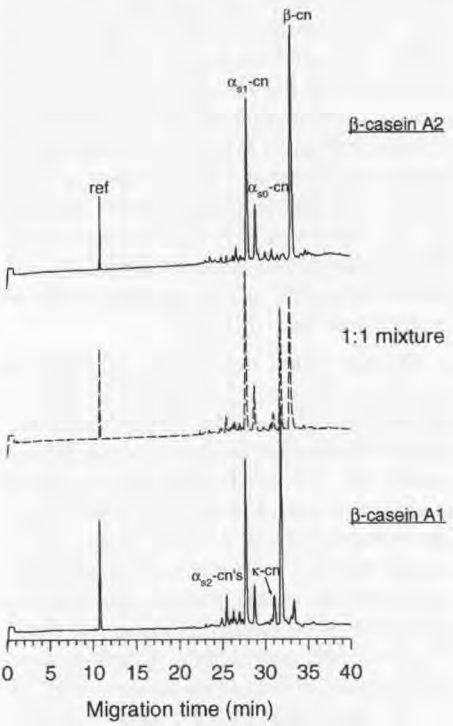

Fig 2. Capillary electropherograms of acid casein containing only β-casein A1 and β-casein A2 respectively, and of a 1:1 mixture of the two samples (dashed line). Separations were performed in a 58.5-cm long coated capillary, at 20 kV (~ 65 μA); ref: reference compound (Lys-Tyr-Lys).

Électrophérogrammes capillaires des précipités acides de caséine contenant les caséines β A1 ou A2 seulement, et d'un mélange des deux échantillons (ligne pointillée). La séparation est réalisée avec un capillaire traité de 58,5 cm à 20 kV (~ 65 μA) ; ref : composé de référence (Lys-Tyr-Lys).

at low pH. This is in contrast to the analysis at high pH, where the two β-casein variants have the same net charge and migrate as a single zone (Kristiansen et al, 1994).

Application to caseins from cheeses

Isoelectrically precipitated caseins

When applied to caseins from cheeses, different profiles were obtained from UF-Feta and Danbo (fig 3). The casein pattern of the UF-Feta cheese (fig 3, left) looked very much like that for acid casein (fig 1), as only limited proteolysis occurred during manufacture and storage of this cheese, and the peaks for the α_{s1}-, α_{s0}- and β-caseins were immediately recognisable. The UF-Feta cheese was manufactured from bulk milk; accordingly, distinct peaks for the two dominating β-casein variants A1 and A2 were visible. The peak just before β-casein A1 contained κ-casein, as shown by analysis of the Feta sample spiked with κ-casein (not shown).

The electrophoregram of the caseins from the Danbo cheese (fig 3, right) was somewhat more complicated due to the presence of a number of hydrolysis products. Comparison with the analysis of pure β-casein showed that the peaks at ~25 and ~26 min were due to β-casein A1 and A2, respectively. Other major intact caseins were not positively demonstrated. The casein degradation products most often found in various cheeses stem from the chymosin-catalysed hydrolysis of κ- and α_{s1}-casein and from the plasmin catalysed-hydrolysis of

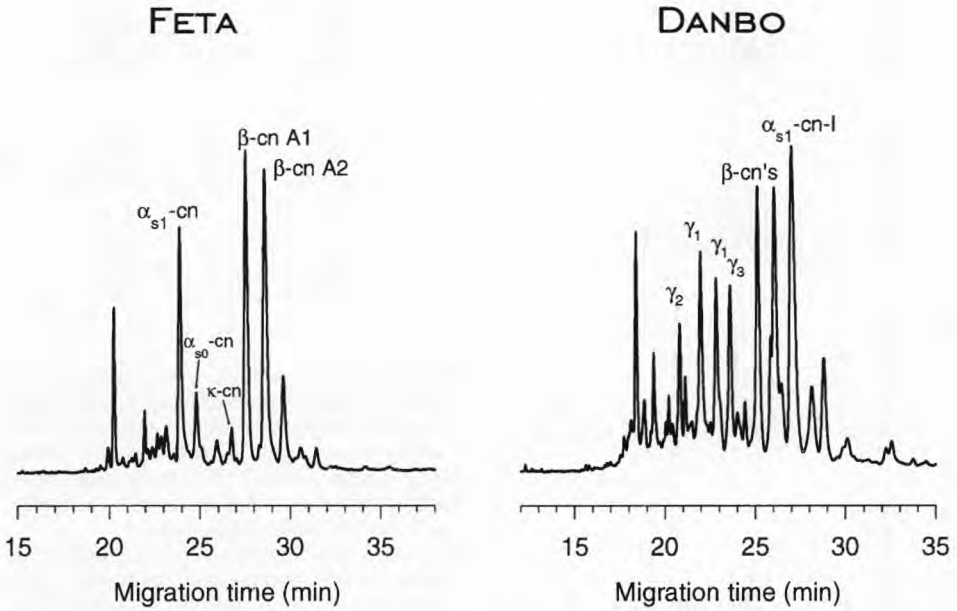

Fig 3. Capillary electrophoresis of caseins from UF-Feta (6 weeks old) and Danbo (12 weeks old) cheeses. Capillary electrophoresis conditions as described in the legend for figure 1.
Électrophorèse capillaire des caséines d'un Feta-UF (âgé de 6 semaines) et d'un Danbo (âgé de 12 semaines). Les conditions d'électrophorèse sont les mêmes que celles décrites sous la figure 1.

β-casein (table I) (Christensen et al, 1989; Creamer, 1991; Calvo et al, 1992; Centeno et al, 1994). In order to identify these major degradation products, hydrolysis of individual caseins with the relevant enzymes was performed (fig 4).

α_{s1}-Casein with added chymosin, both in solution and in cheese, is rapidly hydrolysed at the carboxylic side of Phe23 or Phe24 to give the soluble peptide f1–23/24 and the pH 4.6 insoluble peptide, f25–199, named α_{s1}-casein-I (Mulvihill and Fox, 1979; Grappin et al, 1985; Kristiansen et al, 1994). Accordingly, Figure 4A shows that the peak for α_{s1}-casein (and for α_{s0}-casein) disappeared upon the action of chymosin on α_s-casein in solution, resulting in appearance of a new peak with higher t_m in the electropherograms (and a minor peak with even higher t_m). These new peaks are believed to represent the C-terminal fragment (f25–199) of α_{s1}-casein and α_{s0}-casein, respectively. The migration time of the peak

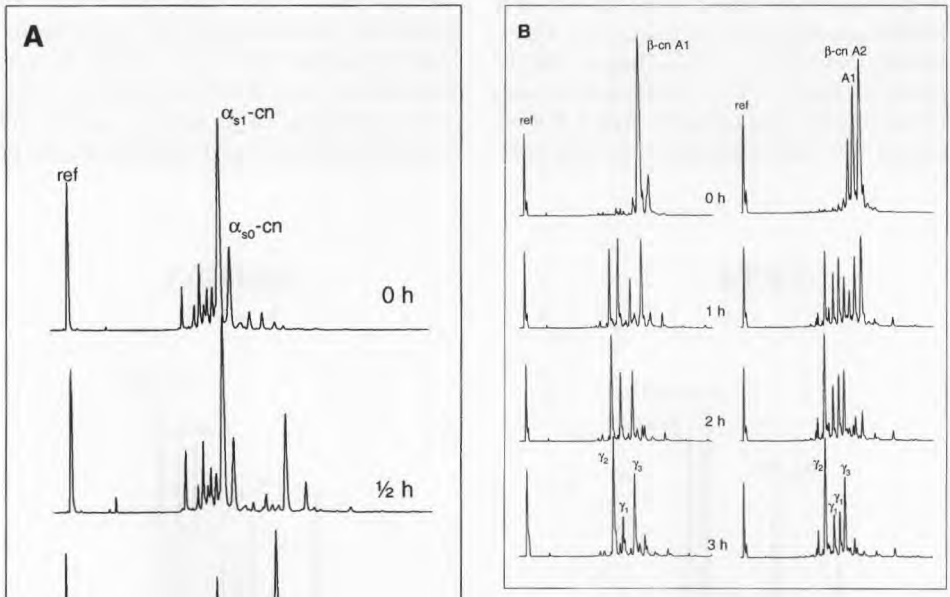

Fig 4. Capillary electrophoresis of samples from isolated casein fractions taken at different intervals after addition of protease. **A**. α_s-Casein hydrolysed by chymosin. **B**. β-Casein A1 (left) and β-casein variants from bulk milk (right) hydrolysed by plasmin. CE conditions as for figure 1; ref: reference compound.

Électrophorèse capillaire des échantillons de caséines isolées prélevés à différents temps après l'addition de la protéase. ***A****. Caséine* α_s *hydrolysée par la chymosine.* ***B****. Caséine βA1 (à gauche) et caséine β du lait cru (à droite) hydrolysées par la plasmine. Conditions d'électrophorèse identiques à celles de la figure 1 ; ref : composé de référence.*

for α_{s1}-casein-I, calculated relative to the reference compound (Lys-Tyr-Lys) and compared to the relative migration time of the β-caseins suggests that the α_{s1}-casein-I has a lower mobility than β-casein A2. Thus, the peak at 27 min in figure 3 should be α_{s1}-casein-I. This was further confirmed by analysis of a mixture of α_{s1}-casein hydrolysed for 4 h (mainly consisting of α_{s1}-casein-I) and casein from Danbo cheese, as only one single peak appeared after the peak for β-casein A2. The small peak next to the β-casein A2 in the electrophoregram from the Feta cheese (t_m ~29.7 min; fig 3, left) likewise represents α_{s1}-casein-I.

Figure 4B (left) shows that upon incubation with plasmin, the β-casein A1 was quickly hydrolysed to yield three main components (2 h). According to Swaisgood (1992) these are the so-called γ-caseins (see table I). It seems that upon further incubation, the second of the γ-caseins was gradually transferred into the two other γ-caseins. This can be explained if the second γ-casein is γ_1-casein (f29–209). By splitting off the fragments f29–105 and f29–107 respectively from the γ_1-casein, the γ-caseins γ_2 and γ_3 result. γ_2-Casein contains His and Lys at positions 106 and 107, which are positively charged at low pH, and thus has the highest mobility under the present conditions. Therefore, the order of migration times of the three γ-caseins must be $\gamma_2 < \gamma_1 < \gamma_3$.

When β-casein from bulk milk was used as a substrate for plasmin, four components resulted (fig 4B, right, 2 h), of which the two with intermediate migration were further degraded during longer incubation with plasmin. As the difference in the primary sequences of β-casein A1 and A2 is in position 67 (His and Pro, respectively), a position only contained in γ_1-casein, the results are in agreement with formation of two different γ_1-caseins, one from each of the β-caseins A1 and A2, but only one γ_2- and one γ_3-casein.

The Danbo cheese (fig 3, right) contains both the A1 and the A2 variants of β-casein, and thus four γ-caseins should result from their partial degradation. By comparison of relative migration times of the peaks in the electropherograms from the Danbo cheese and the γ-casein hydrolysates, the β-caseins were identified as shown in figure 3.

Analysis of α_{s1}-casein mixed with γ-caseins from β-casein A1 showed that α_{s1}-casein and γ_1-casein A1 were co-migrating (results not shown). However, a slight increase in pH and buffer concentration changed the relative mobility of the α_{s1}-casein and the γ-caseins (not shown). Thus, due to the high efficiency of CE, it should be possible to obtain a separation of all five components by slight modification of the pH. Otherwise the degradation of α_{s1}-casein can be deduced from the appearance of α_{s1}-casein-I.

Use of untreated capillary

All results reported till now were obtained using the CElect P1 coated capillary according to de Jong et al (1993). When many cheese samples were run in series, sometimes splitting of all peaks occurred (results not shown), a phenomenon that could not be avoided unless the capillary was replaced by a new one. As untreated capillaries are much cheaper than the coated capillaries, it was preferable to use the untreated capillaries. At pH 2.5, the silanol groups of the untreated capillary should be protonated, preventing the positively charged caseins from interfering with the capillary wall. We expected therefore that the separation would not be significantly deteriorated by changing to an untreated capillary. A comparison of electropherograms of selected samples run in the coated and the untreated capillary, respectively, is shown in figure 5.

In fact, very similar casein patterns were obtained when caseins from Mozzarella cheeses were analysed using the CElect P1

and the untreated capillary (fig 5). This was also the case when a sample of acid casein was analysed (results not shown). A higher absolute absorbance was obtained with the coated capillary, otherwise the relative height of the peaks for the major caseins seemed to be identical with the two capillaries (compare fig 5A/B and C/D, respec-

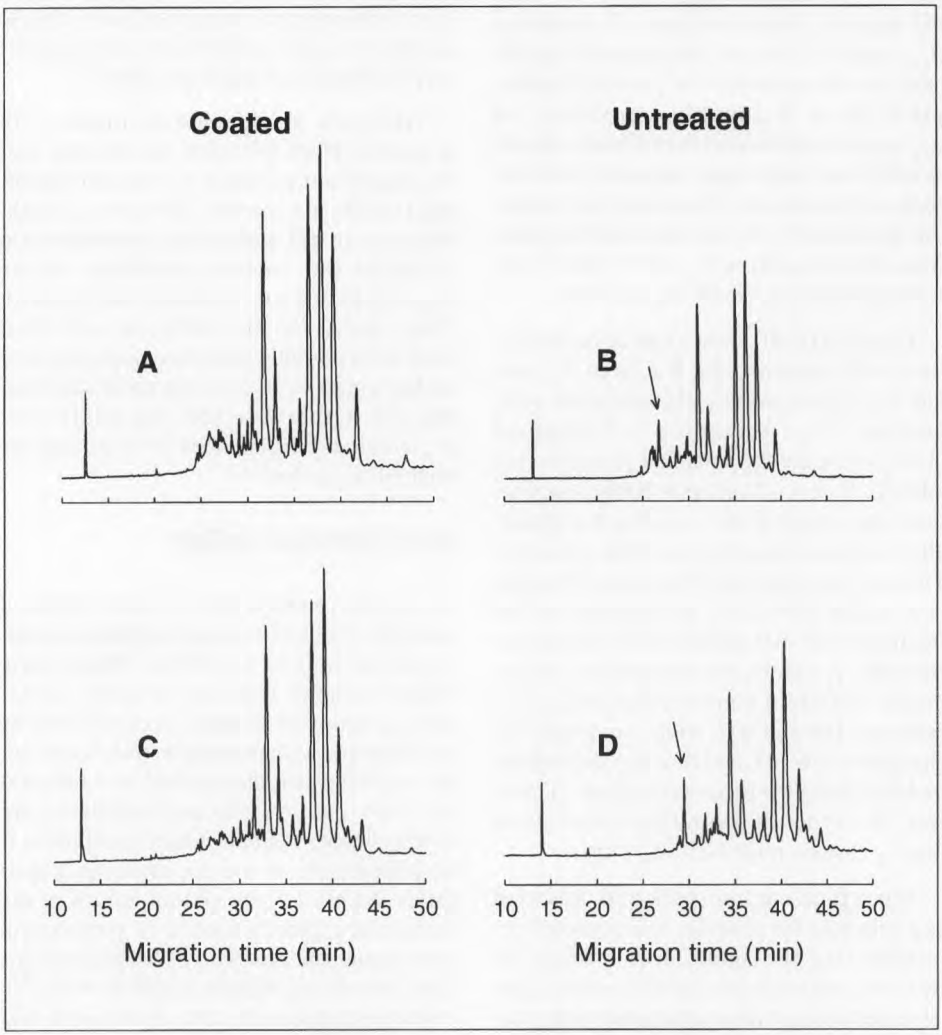

Fig 5. Capillary electrophoresis of caseins from Mozzarella cheeses using a hydrophilically coated capillary (**A** and **C**) and an untreated capillary (**B** and **D**), respectively. **A**, **B**: 1 week old; **C**, **D**: 5 weeks old. Capillary electrophoresis conditions, coated capillary: 52 cm, 13.5 kV (~ 36 μA), 0.02% HPMC; untreated capillary: 60 cm, 17 kV (~ 36 μA), 0.02% HPMC. Arrows indicate para-κ-casein.
*Électrophorèse capillaire des caséines des fromages de Mozzarella avec un capillaire traité (**A** et **C**) et un capillaire non traité (**B** et **D**), respectivement. **A**, **B** : Mozzarella âgée d'une semaine ; **C**, **D** : âgée de 5 semaines. Conditions d'électrophorèse capillaire traité : 52 cm, 13,5 kV (~ 36 μA), 0,02 % HPMC, capillaire non traité : 60 cm, 17 kV (~ 36 μA), 0,02 % HPMC. Les flèches indiquent la caséine para-κ.*

tively). The identification of major caseins and degradation products presented above for the coated capillary, therefore is considered to be valid for the untreated capillary also, the four highest peaks representing α_{s1}-casein, β-casein A1, β-casein A2 and α_{s1}-casein-I, respectively. The only clearly visible difference between electrophoregrams obtained with the two capillaries was that a peak appeared in the first part of the electrophoregrams with the untreated capillary, but not with the coated capillary (arrows in fig 5). This peak, however, had a much higher electrophoretic mobility than the α_{s1}-casein, which is the first migrating major casein, and also higher than the γ-caseins, and thus should not interfere with the determination of the major caseins and their degradation products. By mixing the Mozzarella sample with a sample of chymosin-treated κ-casein, this peak was shown to represent para-κ-casein (table I). The narrow peaks at ~20 min in the electrophoregrams from the Feta and at ~18 min in the electrophoregrams from the Danbo cheese (fig 3) very likely also represent para-κ-casein. The latter results, however, were obtained with the coated capillary, indicating that the absence of para-κ-casein in the Mozzarella cheeses analysed with the coated capillary (fig 5) is not due to this κ-casein fragment interacting with the hydrophilic groups of the coated capillary. The casein profiles of Mozzarella cheeses using the coated capillary were obtained shortly after addition of sample buffer, whereas the analyses using the untreated capillary were performed at a later date, suggesting that para-κ-casein was only slowly liberated by disulfide reduction, resulting in increased concentration with increasing time after addition of sample buffer (compare the section on varying sample preparation procedure below).

An example of application of the casein analysis method with an untreated capillary to follow the casein degradation during limited cheese maturation is shown in figure 6. Although the proteolysis rate of Mozzarella

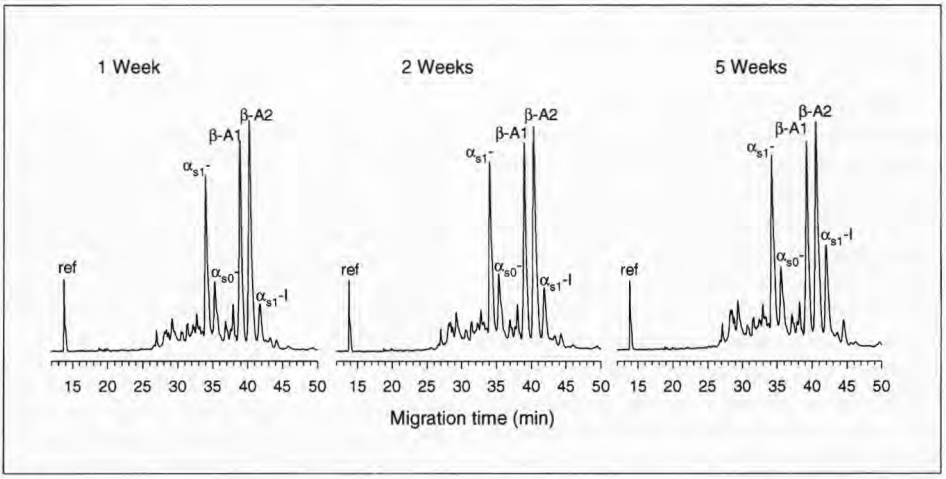

Fig 6. Capillary electrophoresis of caseins from UF-Mozzarella cheeses stored at 5 °C for 1 to 5 weeks. Untreated capillary, 17 kV (~ 35 μA); ref: reference compound.
Électrophorèse capillaire des caséines de Mozzarella-UF conservée à 5 °C pendant 1 à 5 semaines. Capillaire non traité, 17 kV (~ 35 μA) ; ref : composé de référence.

cheese is very low and the present cheeses were made with the inclusion of whey proteins which generally retard the maturation process, a noticeable increase in the concentration of the α_{s1}-casein-I was seen during the few weeks of storage at 5 °C (fig 6). Surprisingly, the peak for α_{s1}-casein did not decrease accordingly, perhaps because a small fraction of the β-casein had been transformed into γ_1-casein, which has a mobility similar to α_{s1}-casein under the present conditions.

Probably much more dramatic changes in the casein pattern would be revealed if analysis were performed on cheeses that require maturation, eg, Danbo or Gouda, as has been shown previously by use of urea–PAGE (Visser and de Groot-Mostert, 1977).

Varying sample preparation procedure

The cheese samples treated above were all prepared by acid precipitation of the caseins from a citrate suspension of the cheese. In order to examine whether the sample preparation procedure could be simplified by omission of the precipitation step, analysis of variously treated citrate suspensions of UF-Feta was performed.

When the citrate solution of the UF-Feta cheese was analysed without addition of sample buffer, peaks for the major caseins were recognisable in the electrophoregram, but they were broad and insufficiently separated and had a low number of theoretical plates (results not shown). Dilution of the sample with water (1:1) only gave a limited improvement. However, when the citrate solution was diluted (1:1) with sample buffer, the caseins and hydrolysis products were very well separated with similar or higher efficiency than the acid-precipitated caseins (fig 7).

It can be seen from figure 7 that the separation of the caseins in the acid-precipitated sample and in the citrate suspension

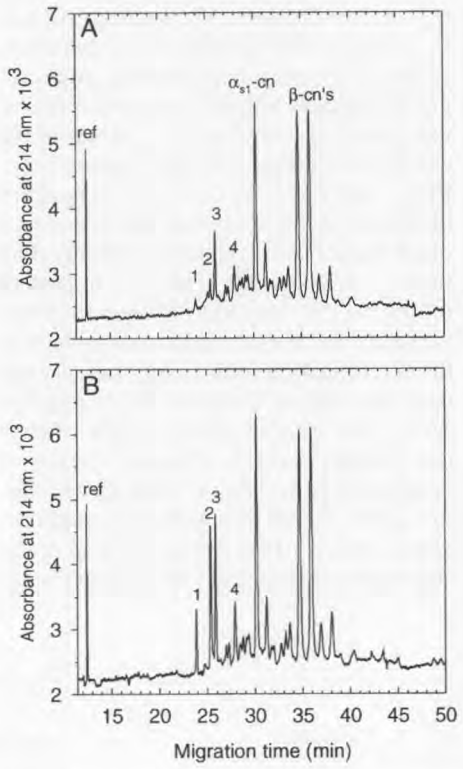

Fig 7. Capillary electrophoresis of caseins from UF-Feta cheese stored at 5 °C for 40 weeks. **A**. pH 4.6 precipitate of citrate solution with added sample buffer (2 mL /mL citrate solution). **B**. Citrate solution diluted 1:1 with sample buffer. Untreated capillary, 17 kV (~ 40 μA); ref: reference compound. Peak identification: 1, α-lactalbumin; 2, β-lactoglobulin; 3, para-κ-casein; 4, α_{s2}-casein.

*Électrophorèse capillaire des caséines d'un Feta-UF conservé à 5 °C pendant 40 semaines. **A**. Précipité à pH 4,6 de la solution de citrate, avec le tampon pour échantillon ajouté (2 mL/mL solution citrate). **B**. Solution de citrate dilué 1:1 avec le tampon pour échantillon. Capillaire non traité, 17 kV (~ 40 μA) ; ref : composé de référence. Identification de pic : 1, α-lactalbumine ; 2, β-lactoglobuline ; 3, caséine para-κ ; 4, caséine α_{s2}.*

diluted with sample buffer was almost identical, and the peak widths were very similar. The slightly depressed peak heights of the caseins in the acid-precipitated sample (fig 7A) in comparison with the unprecipitated sample (fig 7B) might be due to inaccuracies in pipetting small volumes. One noticeable difference between the electropherograms shown in figure 7, however, was the occurrence of four narrow but significant peaks in the first part of the electropherogram from the unprecipitated sample (fig 7B), components with higher mobility than the caseins. However, these peaks, designated 1 to 4, can also be distinguished in the electropherogram from the acid-precipitated sample (fig 7A), but at much lower intensity. They were not present in the electropherograms of citrate solutions without sample buffer, in which only one peak of the same height as peak 1 was seen but at a migration time of 20 min (results not shown). This suggests that the components 1 to 4 were associated with each other in the citrate solution, but became dissociated upon addition of urea and DTE. The low concentrations of these components in the pH 4.6-precipitated cheese sample can be explained if the associated molecules are partly soluble at pH 4.6, or not fully dissociated upon addition of sample buffer. By mixing the unprecipitated cheese sample with relevant amounts of rennet whey and hydrolysed κ-casein respectively, the three first peaks were identified as α-lactalbumin, β-lactoglobulin and para-κ-casein, respectively. Peak 4 has the same relative t_m as α_{s2}-casein. Due to their content of cysteic residues the β-lactoglobulin, α_{s2}-casein and κ-casein/para-κ-casein are able to form disulfide bonded aggregates (Elfagm and Wheelock, 1978). α-Lactalbumin also contains eight Cys residues, but all are engaged in disulfide bridges. Probably –SH/S–S interchange reactions had occurred between the four proteins during the short heat-treatment of the milk before cheese formation.

These results indicate that the simpler sample preparation is a precondition for obtaining quantitative results on the para-κ-casein, and suggests that trials with varying concentrations and varying times of incubation with urea and DTE should be performed in order to ensure that all aggregates are solubilised.

The complementary part of κ-casein formed by the chymosin catalysed cleavage of κ-casein, the caseinomacropeptide (f106–169; Otte et al, 1995) might also be present in some cheeses, eg, fully concentrated UF-cheeses. As this peptide is soluble at pH 4.6, it is present in the unprecipitated samples of such cheeses. Analysis of a caseinomacropeptide sample in the untreated capillary, however, showed that this peptide has a very low mobility under the present conditions and was migrating far behind the α_{s1}-casein-I. Thus, omission of the acid precipitation step in the sample preparation procedure should not result in the presence of any major compound that would interfere with the determination of the major caseins and hydrolysis products.

Performance of the method

The efficiency of the method was high, giving theoretical plate numbers for the major casein peaks in acid casein and acid precipitated cheese casein of 100 000–300 000 per metre, depending on the sample composition, dilution and the state of the capillary. The plate numbers obtained with the untreated capillary and the coated capillary for the individual caseins in the Mozarella samples (fig 5) were very similar, only slightly higher with the untreated capillary. The proteins in the rennet whey sample gave peak efficiencies exceeding 300 000 theoretical plates/m. These values are a little lower than the efficiency obtainable by use of a citrate buffer as the CE electrolyte (de Jong et al, 1993), but still sufficient to give complete resolution of the caseins.

The repeatability of the method in the coated and the untreated capillary was also very similar (table II). With both capillaries there was a good repeatability on the relative migration times, ~0.5% relative standard deviation. However, the repeatability of the peak areas and normalised peak areas was not satisfactory. The relative standard deviations (RSD) of 6–8% gave only a two-fold improvement over the repeatability obtainable by scanning of electrophoresis gels (Madsen JS, pers commun). These high RSD values might be due to lack of temperature control and/or extensive capillary wash between runs, as it is not possible with the Quanta 4000 to control the temperature of the capillary and to automatically rinse the capillary with base without changing the outlet buffer. An experiment performed with other equipment (HP G1602A HPCE system, Hewlett–Packard A/S, Waldbronn, Germany) at a constant outer capillary temperature of 30 °C, and with extensive capillary wash between injections (NaOH for 2 min, followed by water for 2 min and finally buffer for 3 min) gave an RSD value of 2.7% ($n = 9$) for the normalized peak area of β-casein A1 in a UF-Feta cheese sample. This value is similar to the values obtained by de Jong et al (1993) for caseins in milk.

Table II. Repeatability expressed as relative standard deviation of migration times and peak areas from nine analyses of acid-precipitated casein using a hydrophilically coated and an untreated capillary, respectively. The values presented (expressed in %) are mean values for the peaks for β-casein A1 and α_{s1}-casein.
Répétabilité exprimée comme déviation standard relative des temps de migration et des aires de pic pour neuf analyses de caséine acide avec un capillaire traité par un polymère hydrophile et un capillaire non traité, respectivement. Les taux présentés (exprimés en %) sont les moyennes pour les pics de caséine βA1 et de caséine α_{s1}.

Parameter	*Coated*	*Untreated*
Migration time	1.9	1.9
Relative migration time [a]	0.4	0.6
Peak area	8.2	7.0
Normalised peak area [b]	7.0	5.6
Peak height	0.7	3.7

[a] Relative to the reference compound. [b] Peak area divided by migration time.
[a] *Relatif au composé de référence.* [b] *Aire de pic divisé par temps de migration.*

The performance of the untreated capillary was further studied with respect to linearity of the detector response vs injection time and sample concentration, respectively. The injection linearity was satisfactory over almost one decade of injection time (5 to 40 s) with $r^2 > 0.996$ (not shown). A linearity plot of peak area/injection time vs injection time (Dorschel et al, 1989) showed a true linearity for α_{s1}- and β-casein within the 10–40-s interval with a tolerance of ± 3% (not shown).

The correlation between α_{s1}-casein or β-casein A1 concentration in standard samples and resulting peak area in the electrophoregrams was almost linear (fig 8A). The linearity plots (peak area/concentration vs concentration) show that all sensitivity values were close to the interval representing 95% to 105% of the mean value (dotted lines in fig 8B). Use of corrected peak areas (area divided by migration time) instead of simple peak areas did not change the linearity values.

Provided that the recovery of caseins in different cheese matrices is satisfactory the CE method presented, with improved repeatability on peak areas and with assessment of necessary reductant concentration and reaction time, might be used to quantitatively follow the breakdown of all major caseins and the formation of primary degradation products in cheese. The only exception is α_{s1}-casein in cases where γ_1-casein is being formed.

CONCLUSION

The four different bovine caseins were well separated by CE in coated and untreated capillaries under the present conditions using HPMC as the polymeric additive. Further, at least two genetic variants of β-casein, A1 and A2, were separated as were the two phosphorylation stages of α_{s1}-casein and four phosphorylation stages of the α_{s2}-casein monomer.

The major degradation product of α_{s1}-casein formed by the action of chymosin, α_{s1}-casein-I, was separated from α_{s1}-casein and from all other major caseins in the cheeses examined. The major degradation products of β-casein with plasmin, γ-caseins, were migrating well ahead of the β-caseins but in the area of the α_s-caseins. The two peptides resulting from the chymosin catalysed cleavage of κ-casein, para-κ-casein and caseinomacropeptide, migrated in front of and behind the intact caseins, respectively, and did not interfere with other caseins. Thus, in the absence of β-casein degradation, all major casein components in cheese should be determinable in one analysis.

The repeatability of the migration time calculated relative to the migration time of a reference compound was good. With minor improvements and validation, the CE method presented might be used for quantitative assessment of casein degradation in cheeses. Furthermore, the extraction procedure for cheeses, including UF-cheeses and cheeses containing denatured whey proteins, can be simplified by use of the citrate solution of the cheese without acid precipitation of the caseins.

The CE method presented is considered to be superior to urea–PAGE and an attractive alternative or supplement to existing

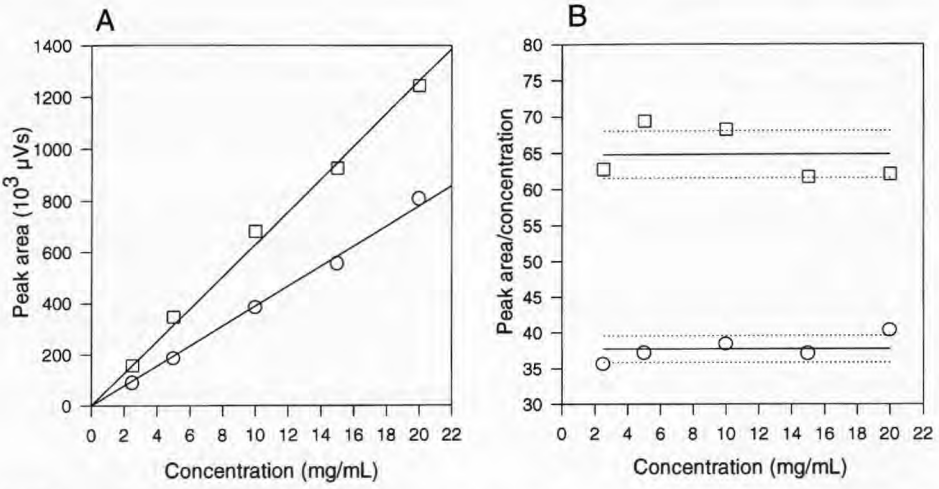

Fig 8. Linearity of the method assessed with purified caseins using an untreated capillary. **A**. Peak area as function of concentration of caseins. **B**. Linearity plot of the data in A. □: β-casein A1, ○: α_{s1}-casein. Other conditions as described in the Methods section.
*Linéarité de la méthode avec des caséines purifiées et un capillaire non traité. **A**. Aire de pic en fonction de la concentration de caséine. **B**. Courbe de linéarité des données montrées en **A**. □: caséine β A1 ; ○: caséine α_{s1}. Autres conditions : comme décrites dans la section 'Méthodes'.*

HPLC methods for determination of proteolysis in cheese.

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