



ORIGINAL
RESEARCH

Validation of a reversed-phase high-performance liquid chromatographic method for the quantification of primary proteolysis during cheese maturation

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A new fast and reliable reversed-phase high-performance liquid chromatographic method is proposed for quantifying all the major casein fractions during cheese proteolysis as an alternative to more complex methodologies. Excellent separation of the caseins, para- κ -casein (ρ - κ -CN), α_{s1} -casein (α_{s1} -CN), α_{s1} -I-casein (α_{s1} -I-CN), α_{s2} -casein (α_{s2} -CN), and β -casein (β -CN) was achieved, in terms of linearity, reproducibility, repeatability, accuracy and certified standard recovery. Validation by quantification of semihard and Maasdam-style cheeses gave comparable results for ρ - κ -CN, β -CN and α_s -casein (α_s -CN) to published data for both urea-polyacrylamide gel electrophoresis and capillary electrophoresis.

Keywords Casein fractions, Characterisation, Chromatography, Cheese.

INTRODUCTION

The three main casein subgroups in milk are α_s -CN, β -CN and κ -casein (κ -CN). The action of chymosin (an aspartyl proteinase, E.C. 3.4.23.4) during cheese manufacture hydrolyses the Phe₁₀₅ – Met₁₀₆ bond of κ -casein (Van Hooydonk *et al.* 1984) resulting in the release of the negatively charged water-soluble C-terminal caseino-macropeptide (CMP) or glycomacropeptide into the whey, initiating milk coagulation (Calvo *et al.* 1995; McSweeney and Fox 2013). The remaining κ -casein component is insoluble and remains in the cheese curd and is referred to as ρ - κ -CN.

Proteolysis is a major component of cheese maturation, influencing both the development of texture and flavour as ripening progresses. It is typically characterised as two distinct processes: primary proteolysis and secondary proteolysis. Primary proteolysis is the process where caseins undergo the initial hydrolysis by chymosin and indigenous milk enzymes such as plasmin and cathepsin D (McSweeney and Fox 2013), and secondary proteolysis is the process where products of primary proteolysis

are further hydrolysed into peptides and amino acids by the activity of proteases and peptidases from starter and nonstarter bacteria (Sousa *et al.* 2001).

In defining cheese as a protein matrix entrapping moisture, fat, minerals, etc. (Hickey *et al.* 2018), proteolysis (in part) of this matrix is a key index of cheese maturation, which results not only in softening of texture but also in release of peptides and amino acids that are important for taste. These peptides and amino acids are also further catabolised to produce volatile aromatic compounds that also influence flavour. Quantification of proteolysis is thus an important measure of the degree of ripening or maturity and is also strongly linked to the quality of the cheese.

Methods for measurement of proteolysis in cheese may be characterised as follows:

- i) those that quantify the concentration of nitrogen fractions (usually by Kjeldahl) and solubilised nitrogen in solutions such as water, citrate solutions, water adjusted to pH 4.6, trichloroacetic acid, phosphotungstic acid and ethanol (Tieleman and Warthesen 1991; Bütikofer *et al.* 1993).

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- ii) those that quantify the amino groups of the free amino acids through reaction with compounds such as cadmium ninhydrin or trinitrobenzenesulphonic acid and measurement using spectrophotometric methods (Kuchroo *et al.* 1983; Folkertsma and Fox 1992; Bouton and Grappin 1994).
- iii) Separation, identification and quantification of caseins by chromatography: This involves the separation of the cheese into water-soluble and water-insoluble fractions, removal of fat and dissolution of the insoluble protein phases in urea. Separation of the caseins may be achieved by using high-performance liquid chromatography (RP-HPLC) or ion exchange by protein liquid chromatography (PLC). In a previous study, Christensen *et al.* (1989) analysed lyophilised cheese powder samples using RP-HPLC. This study involved a number of complex sample preparation steps, including centrifugation, pH adjustment, dialysis and lyophilisation before final resuspension in a concentrated urea solution containing dithiothreitol (DTT).
- iv) Electrophoresis: This involves separation of caseins and casein fractions based on charge and electrophoretic mobility. It provides a visual illustration of individual caseins and some of their main degradation products. Quantification of changing casein fraction concentrations may be achieved by undertaking densitometric scans of the gels. Commercial gel kits are now available to remove background staining, increase sensitivity, reduce staining and destaining times, eliminate the use of some toxic solvents and reduce human error. However, the ingredients of many of these kits are proprietary and are not standardised between manufacturers (Schröder *et al.* 2008; Dyballa-Rukes and Metzger 2009; Beer and Speicher 2019).
- v) Capillary electrophoresis (CE): CE separates components using an electrical field but within the confines of a narrow tube (Whatley 2001). A number of studies have applied CE to profile caseins and their degradation products (Recio *et al.* 1997; Alves *et al.* 2013; Bijl *et al.* 2014; Delgado *et al.* 2015; Taivosalo *et al.* 2018).

Given that indices of proteolysis are used as key indicators of quality and age in cheese, it is important to have robust methodology to accurately quantify the main casein fractions resulting from the action of indigenous and exogenous proteinases in order to elucidate subtle changes that occur over maturation. To date, no single chromatographic procedure exists that can accurately quantify α s1-, α s1-1-, α s2-, β - and p-k-CN simultaneously in the insoluble fraction of cheese.

The objective of this study was to develop and validate a fast and economic high-performance liquid chromatographic method, which could be used to separate and quantify the primary casein proteolysis products of the water-insoluble part of semihard and hard cheeses.

MATERIALS AND METHODS

Materials

Casein and whey standards (κ -CN, α s-CN, β -CN, α -lactalbumin and β -lactoglobulin A and B) for the RP-HPLC were supplied by Merck (Sigma-Aldrich, Arklow, Co. Wicklow, Ireland). A certified skim milk powder standard (BCR-063R) was supplied by the Reference Materials Unit (European Commission, Directorate General Joint Research Centre, Geel, Belgium). HPLC grade acetonitrile, spectroscopic grade trifluoroacetic acid (TFA), guanidine, bis-tris propane and DTT were supplied by Merck (Sigma-Aldrich, Ireland). Bulk raw milk samples were obtained from the Teagasc Animal and Grassland Research Innovation Centre Moorepark, Fermoy, Co Cork, Ireland (AGRIC), on alternative days for method validation. All milk samples were analysed on the day of collection to avoid microbial spoilage.

Cheese manufacture, maturation and sampling

A semihard continental cheese type was manufactured as described by Hickey *et al.* (2018) using standardised and pasteurised cheese milk inoculated with *Streptococcus thermophilus* (0.01% w/v) and *Lactobacillus helveticus* (0.005% w/v) sourced from Chr. Hansen Ltd (Little Island, Co Cork, Ireland). Chymosin (Chy-Max Plus ~200 IMCU/mL) (Chr. Hansen Ltd, Ireland) was used as a coagulant, and the curds were cooked to a maximum scald of 40 °C. The cheeses were subsequently brine-salted and ripened at 8 °C for 186 days.

Two Maasdam cheeses were produced 2 weeks apart, also from standardised, pasteurised milk. Starter cultures (Chr. Hansens Ltd, Ireland) were comprised of a DL-type culture with *Lactobacillus helveticus* as an adjunct. Chymosin was used as coagulant, and the cheeses were manufactured as described by Lamichhane *et al.* (2018). After brining, the cheeses were dried, vacuum-packed and ripened at 10 °C for 10 days, 20 °C for 30 days and subsequently at 2 °C for a further 190 days. The semihard cheese was sampled at 7, 28, 56, 98 and 186 days of maturation, while the Maasdam cheeses were sampled at 0, 56, 111, 164 and 227, or 0, 60, 99, 152 and 215 days of maturation.

Chemical composition

At 14 days post-manufacture, grated cheese samples were analysed for moisture (ISO 5534), salt (ISO 5943), protein (ISO 8968) and fat (ISO 1211). The pH was measured on cheese slurry prepared from 20 g of grated cheese combined with 12 g of H₂O (BSI 1976) at the various sampling points throughout maturation.

Cheese characterisation using RP-HPLC

For the characterisation of the cheese, between 300 and 500 mg of cheese pellet was accurately weighed out and solubilised in a 7 M guanidine buffer containing DTT and

bis-tris-propane at pH 7.0 (Bobe *et al.* 1998), heated to 60 °C in a water bath, mixed using an Ultra-Turrax mixer (IKA-Werke, GmbH, Staufen, Germany) and incubated at room temperature for 1 h. The samples were then diluted 1:3 in 4.5 M guanidine buffer to a final concentration of between 2.4 and 4.5 mg/mL of total protein as described by Bobe *et al.* (1998) before filtration through a 0.45- μ m cellulose filter (Macherey-Nagel GmbH, 6-8 Neumann-Neander Str., Germany) into a screw-capped 2-mL vials (Carl Stuart Limited, Whitestown, Dublin 24, Ireland). A 25 μ L volume was then injected onto the HPLC system.

An Agilent 1200s HPLC equipped with a quaternary pump, heated column compartment, temperature-controlled autosampler and multiwavelength detector was used with the data being processed using the ChemStation software (Agilent Technologies Ireland Ltd, Little Island, Cork, Ireland). Separation was achieved using an Aeris C4 column (4.6 mm \times 250 mm; Phenomenex Macclesfield, Manchester, UK), with a flow rate of 0.8 mL/min at 35 °C. Solvent A was an acetonitrile–water–TFA mixture (100:899:1, v/v/v) and Solvent B an acetonitrile–water–TFA mixture (900:99.1:0.9, v/v/v). The solvent conditions were a modification of the gradient first described by Visser *et al.* (1991). The gradient commenced at 26% B, and increased to 37% B at 19 min, to 45% B at 43 min and to 100% B at 49 min where it held for 3 min. The gradient returned to 26% B at 54 min. The total run time was 57 min. Detection of the proteins was at 214 and 280 nm, with quantification at 214 nm. The quantification of the proteins was carried out at 214 nm as this is the standard wavelength used for the absorbance of the peptide bonds found in dairy proteins as used in a number of previous studies (Croguennec *et al.* 2004; Bonfatti *et al.* 2008; O’Kennedy and Mounsey 2009). As one of the purposes of this study was simplification, it is easier and sufficiently accurate using 214 nm alone.

Urea–polyacrylamide gel electrophoresis

Urea–polyacrylamide gel electrophoresis (Urea-PAGE) and RP-HPLC analysis were performed, at time points of 7, 28, 56, 98 and 186 days on the semihard cheese samples and at time points of 0, 56, 111, 164 and 227 days for Maasdam cheese 1 and 0, 60, 99, 152 and 215 days for Maasdam cheese 2, respectively, in order to compare the results for the two methods. A Protean II xi vertical slab gel unit (Bio-Rad Laboratories Ltd., Watford, Herts., UK) and a stacking gel system as described by Andrews (1983) were used. The gels were stained directly by the method of Blakesley and Boezi (1977) using Coomassie Brilliant Blue G250. The samples were visualised by photo scanner (Epson Perfection V700, Westside, London Rd, Hemel Hempstead HP3 9TD, U.K.) using the image analysis software (ImageQuant GE Healthcare, Tullagreen, Carrigtwohill, Co. Cork, Ireland).

Validation of the RP-HPLC method and statistical analysis

Three sets of calibration curves were prepared to establish the linearity of response, coefficients of determination, limits of detection (LOD) and the limits of quantification (LOQ). The analytical performance characteristics of precision, accuracy and the standard recovery for each of the major casein fraction were also investigated using raw milk. This matrix was chosen as it was the starting material for the Cheesemaking processes and has a certified reference skim milk powder standard commercially available, which could then be used for comparative purposes with previous studies (Bordin *et al.* 2001; Bonfatti *et al.* 2008). A semihard cheese and Maasdam cheese were used to validate the procedure (however, the method is applicable to other cheese types). In addition, the cheese extraction process yields a soluble component, which could also be further analysed by RP-HPLC UV/Vis to facilitate peptide profiles (Rohm *et al.* 1996) or by liquid chromatography–tandem mass spectrometry (LC-MS/MS), (Taivosalo *et al.* 2018) to gain a more in-depth analysis of proteolysis over cheese ripening.

The current method proposes a significant simplification of the extraction method of Christensen *et al.* (1989) including removal of time-consuming steps such as dialysis and lyophilisation. The method involves dissolution of cheese samples in a guanidine-based buffer with DTT as the reducing agent. Guanidine and DTT in combination achieve a greater separation of the caseins and a reduction in artefacts upon storage in urea (Bobe *et al.* 1998). A specific fat removal step is not required as this is achieved during filtration, which occurs after reduction in the sample in the guanidine buffer. Homogenisation of the sample is achieved by mixing the sample at elevated temperatures using a high-speed mixer, which does not adversely affect the caseins owing to their inherent heat stability at 60 °C.

A laboratory validation of the RP-HPLC method was undertaken by calculating linearity, precision, accuracy and LOD and LOQ for each of the caseins (p-k-CN, α_{s1} -CN, α_{s2} -CN and β -CN). Three independent sets ($n = 3$) of standard curves at seven different concentrations were prepared for each of the caseins in guanidine and DTT buffer as described (Table 3). The casein standards were prepared separately, and the concentrations of each were as follows: p-k-CN (0.46, 1.15, 2.3, 3.45, 4.6, 6.9 and 9.2); α_{s1} -CN (1.68, 4.2, 8.4, 12.6, 16.8, 25.2 and 33.6); α_{s2} -CN (0.18, 0.45, 0.90, 1.35, 1.80, 2.70 and 3.60); and β -CN (1.57, 3.93, 7.85, 11.8, 15.7, 23.6 and 31.4) μ g. From the calibration curves, the linearity, slope, coefficient of determination (R^2), LOD and LOQ for the system were determined. The LOD was calculated as $(3.3 \times \sigma/S)$, and the LOQ was calculated as $(10 \times \sigma/S)$, where S is the slope of the curve, and σ refers to residual standard deviation of the response (ICH

1995). For this study, the percentage relative standard deviation (RSD %) can be defined as:

$$\text{RSD \%} = (\text{Standard Deviation} / \text{Mean}) \times 100.$$

The precision of the method was measured using the data from both the repeatability and reproducibility data. The interday repeatability of the system was assessed by analysing the RSD % of the concentrations of each of the casein fractions quantified using the same raw bulk milk sample, run ten times consecutively (Table 2). The intraday or total reproducibility compared the RSD % of the concentrations of each casein fraction for the same raw bulk milk sample ran 10 times over 2 days (Table 2). The accuracy of the method was determined by comparing the casein values obtained with those previously reported for milk caseins for the certified reference material BCR-063R (Bordin *et al.* 2001; Bonfatti *et al.* 2008).

The samples were quantified against the known standards for the major caseins (Merck, Sigma-Aldrich, Ireland) using the external standard method (Weston and Brown 1997). The results obtained for the cheeses for α_{s1} -casein, α_{s1-1} -casein, α_{s2} -casein, β -casein and p-k-casein using the RP-HPLC method were calculated for each sample and expressed as percentage of the total casein to aid in comparison with previous studies using hard or semihard cheeses.

RESULTS AND DISCUSSION

Cheese composition

The composition of both semihard and Maasdam cheese types measured in this study is shown in Table 1 and is typical for these cheese types. The semihard cheese is very similar in levels of moisture, moisture in nonfat substance (MNFS), protein, fat, fat in dry matter (FDM), salt, salt in moisture (S/M) and pH measured at 14 days of ripening to an equivalent cheese type reported by Hickey *et al.* (2018). Similarly, the composition and pH of both Maasdam cheeses are similar to those as reported by Lamichhane *et al.* (2018) and Panthi *et al.* (2019).

Validation of the RP-HPLC method

The repeatability and reproducibility of individual and total caseins quantified by the RP-HPLC method are shown in Table 2. The interday repeatability and total reproducibility of the systems were very good with an RSD of $< \sim 1.5\%$ for all of the caseins except for α_{s2} -CN, which was $\sim 5\%$, with the major caseins α_{s1} -CN and β -CN having a RSD of $< 1\%$. The coefficients of determination (r^2) at > 0.99 demonstrated excellent linearity (Table 3). The values obtained from the analysis of the caseins from the certified skim standard (BCR-063R) were in agreement with other studies (Bordin *et al.* 2001; Farrell *et al.* 2004; Bonfatti *et al.* 2008). The values for κ -CN, α_{s1} -CN and β -CN found in the certified standard using this RP-HPLC method were 92.7, 87.8 and

102.5%, respectively and correspond to those reported by Bordin *et al.* (2001).

In order to help visualise changes in the major casein fractions that occur in cheese production, chromatograms derived from both raw milk (not from the same production batches) and ripened cheese samples were overlaid (Figure 1). Although whey proteins are evident in the raw milk chromatograms, the equivalent peaks in the cheese samples were barely perceptible (retention time (RT): 35–40 min), which reflects the loss of the majority of the whey proteins in cheese manufacture (Rynne *et al.* 2004). Similarly, κ -CN (RT: 10–16 min) is present in raw milk samples but absent from the cheese samples, replaced by ρ - κ -CN (RT: 12 min), which reflects the cleavage of κ -CN by chymosin during the coagulation phase of cheese manufacture (Calvo *et al.* 1995; McSweeney and Fox 2013) and the retention of ρ - κ -CN in the curd and partition of the CMP (De Kruif and Zhulina 1996; Wedholm *et al.* 2006, 2008). The effect of proteolysis on the caseins over ripening is also clearly evident in the cheeses (Figure 2) and demonstrates the suitability of the method to easily discern changes over maturation.

The α_{s1} -CN (RT: 24 min) of raw milk is also hydrolysed into: α_{s1-1} -CN (RT: 22 min) and the residual α_{s1} -CN (RT: 24 min), due to the proteolytic action of chymosin; and possibly cathepsin D and plasmin during the Cheesemaking process. The α_{s2} -CN (RT: 17 min) is evident in both the milk and cheese chromatograms (Figure 1). From the chromatogram (RT: 29 min) of the milk (Figure 1), it would appear the two most common genetic variants of β -CN (A^1 and A^2) found in western Holstein cattle are present as previously reported (Visser *et al.* 1991; Bordin *et al.* 2001). A smaller peak appears earlier in the chromatogram just in front of the two main β -CN peaks of bovine milk, which may be the B variant or one of the other genotypes (this would have to be confirmed by mass spectrometry analysis, which was outside the scope of this study). These peaks also appear in the cheese sample (Figure 1); however, the second peak appears larger in comparison with milk possibly reflecting low level proteolysis occurring during the maturation of cheese resulting in the production of the γ -CN. For the purposes of this study, the γ -CN peak was considered as part of the overall β -CN peak although there is scope for further research to clarify this.

Characterisation of the casein fractions in cheese

The concentrations of the various casein fractions, expressed as a percentage of the total caseins (Table 4), are in agreement with previous studies using other methodologies such as sodium dodecyl sulphate (SDS)–PAGE and Urea–PAGE (Andrews 1983; Grappin *et al.* 1999; Soodam *et al.* 2015) and also CE (Vasbinder Astrid and de Kruif 2003; Taivosalo *et al.* 2018). Alkaline gels such as Urea–PAGE do not measure components such as κ -CN and p- κ -CN, as these caseins migrate towards the cathode and are not visualised

Table 1 Composition and pH of a semihard-type and Maasdam cheeses at 14 days of ripening.

Cheese type	Protein (%, w/w)	Moisture (%, w/w)	MNFS ¹ (%, w/w)	Fat (%, w/w)	FDM (%, w/w)	Salt (%, w/w)	S/M (%, w/w)	pH
Semihard cheese	22.65	45.51	62.52	27.20	49.92	1.64	3.61	5.10
Maasdam cheese 1	26.42	41.07	57.17	28.16	47.79	1.27	3.09	5.37
Maasdam cheese 2	27.23	40.95	57.64	28.95	49.03	1.24	3.03	5.31

¹MNFS, moisture in nonfat substance; FDM, fat in dry matter; S/M, salt in moisture.

Table 2 Repeatability and reproducibility of RP-HPLC method in quantifying the major caseins in bulk milk samples expressed as a relative standard deviation per cent (RSD %).

Parameter	Number of samples	κ -CN		α_{S2} -CN		α_{S1} -CN		β -CN		Total CN	
		(g/L)	RSD%	(g/L)	RSD%	(g/L)	RSD%	(g/L)	RSD%	(g/L)	RSD%
Interday repeatability	(n = 10)	5.38	1.48	2.32	5.32	13.2	0.32	11.5	0.74	32.5	0.76
Intraday reproducibility	(n = 20)	5.32	0.98	2.36	5.12	13.0	0.38	11.4	0.53	32.1	0.56

Table 3 Values of components determined for calibration curves of RP-HPLC method; components are as follows: retention time (Rt.), concentration range, coefficient of determination (r^2), slope, limit of detection (LOD) and limit of quantification (LOQ).

Component	Rt (min)	Concentration range (μ g)	r^2	Slope	LOD (μ g)	LOQ (μ g)
ρ - κ -CN	12.8	(0.46–9.20)	0.9982	460.2	0.48	1.45
α_{S2} -CN	17.0	(0.18–3.60)	0.9922	501.7	0.40	1.20
α_{S1} -CN	24.1	(1.68–33.6)	1.0000	878.3	0.16	0.48
β -CN	29.2	(1.57–31.4)	0.9999	1186.3	0.44	1.35

(Grappin *et al.* 1985). Therefore, this method was only used for visual and semiquantitative comparison of some of the individual caseins in these cheese samples. The level of ρ - κ -CN as a percentage of the total casein varied between 12.1% at day 7 and 13.8% at day 186 of maturation in the semihard cheese, and between 11.6 and 13.4% and 11.3 and 13.6% in the Maasdam cheeses. The mean concentrations of ρ - κ -CN throughout maturation were 12.7, 12.9 and 13.2% in the semihard cheese and in Maasdam cheeses 1 and 2, respectively. These values were very similar to those (13.8%) attained for Old Saare cheese at the start of maturation (1 day) as reported by Taivosalo *et al.* (2018) using the CE method.

α_{S2} -CN as a percentage of the total casein levels varied between 12.0% at d 7 and 15.8% at day 98 of maturation for the semihard cheese and between 12.8% and 11.9% at d 0 and 9.8% and 9.3% at d 227 and d 215 for the Maasdam cheeses, respectively.

The mean values over maturation for α_{S1} - and α_{S1-1} -CN combined as a percentage of the total protein recovered from the semihard cheese and both Maasdam cheeses were

38.3, 39.3 and 38.8%, respectively. These concentrations compare favourably with a total α_S -CN content of 38.8% as reported by both Taivosalo *et al.* (2018) for fresh (1 day) Old Saare cheese. Grappin *et al.* (1999) reported combined values for α_{S1} -, α_{S1-1} -, α_{S2} and α_{S1-1} -CN degradation products of 50% total nitrogen in the insoluble phase of 180-day matured Emmentaler cheese and 49% for 180-day Comté cheese by Urea-PAGE, these values are similar to the combined values for α_{S2} -, α_{S1} - and α_{S1-1} -CN for both semihard cheese at 186 days (51%) and Maasdam cheese 1 (47%) at 227 days and Maasdam cheese 2 at 215 days (47%) as determined in this study.

The concentrations of β -CN expressed as a % of the total protein for the semihard cheese varied from 36.1% at day 7 of maturation to 34.9% at day 186. The overall mean values for β -CN for each cheese were obtained by adding the β -CN values for each cheese type and then dividing by the number of days sampled. These values (35.4, 37.6 and 37.6%) in the semihard cheese and in the Maasdam cheeses, 1 and 2 respectively, are also similar to those for intact casein in Old Saare cheese aged from 1 day to

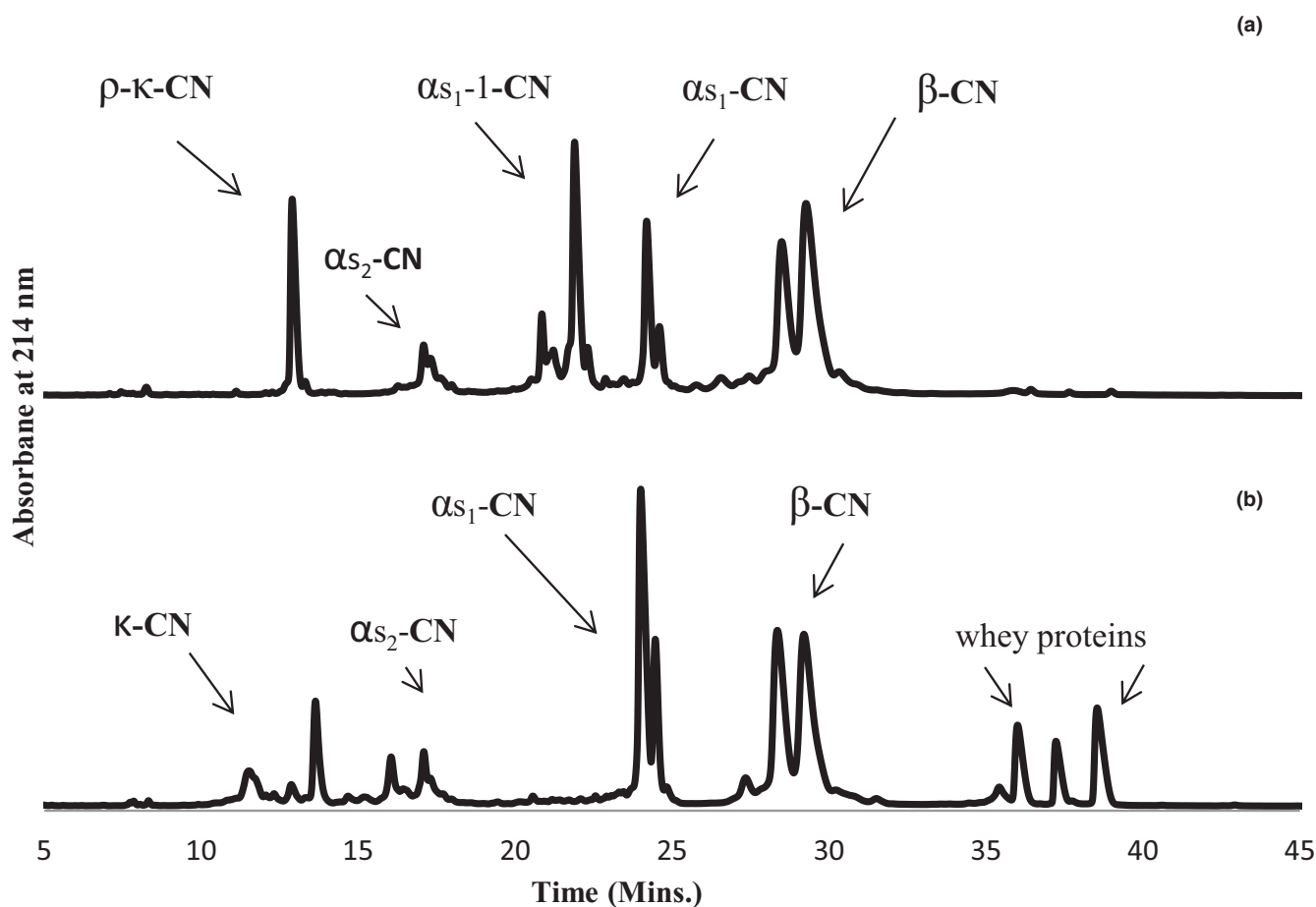


Figure 1 Reversed-phase chromatogram of semihard continental cheese type (a) and raw milk (b) at 214 nm.

180 days as reported by Taivosalo *et al.* (2018). The β -CN values for the semihard cheese (34.9%) at 186 days of maturation and for Maasdam cheese 1 at 227 days (39%) and Maasdam 2 for 215 days (39.4%) were also compared favourably with the % total nitrogen values for the β - and γ -CN in the insoluble phase of Emmental (41%) and Comté (40%) matured over 180 days as reported by Grappin *et al.* (1999).

From the RP-HPLC data, there were a large decrease in the proportion of α_{s1} -CN in both the semihard and Maasdam cheeses and a concomitant increase in the proportion of α_{s1} -I-CN (Table 4). For the semihard cheese, α_{s1} -CN as a percentage of the total casein levels decreased from 20.46% observed on day 7 to only 4.10% at day 186 of maturation, with a drop of $\sim 79\%$. Conversely, the proportion of α_{s1} -I-CN in semihard cheese increased from 19.3% of the total casein on day 7 to 32.2% on day 186, with an increase of $\sim 57\%$. Likewise for the Maasdam cheeses, there was a decrease in the proportion of the α_{s1} -CN from 26.2% to 2.35% in Maasdam cheese 1 and from 28.3% to 1.65% in Maasdam cheese 2 over 227 days and 215 days, respectively. The proportion

of α_{s1} -I-CN increased from 15.9% to 35.3% and from 13.9% to 36.0% for Maasdam cheese 1 and 2, respectively, over the same time period.

During the early phases of maturation, there were a significant hydrolysis of α_{s1} -CN and a concomitant increase in α_{s1} -I-CN (f24-199) for both the semihard (Figure 3) and Maasdam cheese types (Figure 4) as shown by Urea-PAGE. To a lesser extent, there was a progressive but minor accumulation of α_{s1} -CN (f102-199) during maturation. Production of α_{s1} -CN (f24-199) was already well established by day 7 in the semihard cheese, and its accumulation and further hydrolysis continued throughout the 186 days of maturation. Hydrolysis of α_{s1} -CN was at an advanced stage by 56 days, and only traces were remained by 98 days with no further change thereafter, and this is supported by the results from the RP-HPLC (Table 4). The lower levels of α_{s1} -CN in comparison with the original α_{s1} -CN concentration can be attributed to the further degradation of α_{s1} -CN as maturation progressed. For the Maasdam cheeses, there was an even greater hydrolysis of α_{s1} -CN during maturation as seen by the RP-HPLC (Table 4), the majority of

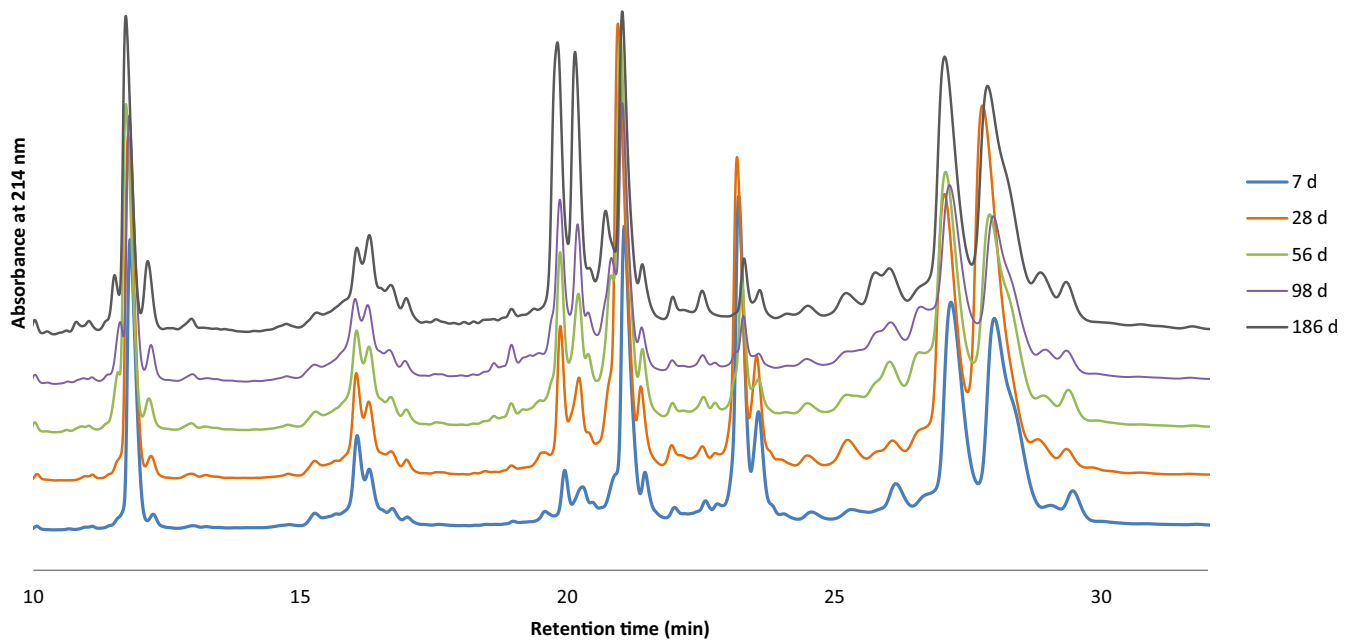


Figure 2 Reversed-phase chromatogram of semihard continental cheese-type maturation over 186 days at 214 nm.

Table 4 Individual casein fractions as measured by RP-HPLC and expressed as a % of total protein for both semihard and Maasdam cheese types \pm standard deviation.

	ρ - κ -CN	α_{s2} -CN	α_{s1} -I-CN	α_{s1} -CN	β -CN
Semihard					
7 days	12.1 \pm 0.29	12.0 \pm 1.11	19.3 \pm 0.41	20.5 \pm 1.43	36.1 \pm 0.31
28 days	11.2 \pm 0.13	12.1 \pm 1.25	25.8 \pm 0.21	14.3 \pm 0.88	36.7 \pm 0.41
56 days	13.0 \pm 0.28	13.6 \pm 0.37	31.4 \pm 0.24	6.95 \pm 0.36	35.0 \pm 0.48
98 days	13.2 \pm 0.06	15.8 \pm 1.06	32.8 \pm 0.46	4.01 \pm 0.33	34.1 \pm 0.29
186 days	13.8 \pm 0.30	15.0 \pm 0.53	32.2 \pm 0.23	4.10 \pm 0.29	34.9 \pm 0.89
Maasdam cheese 1					
0 day	11.6 \pm 0.44	12.8 \pm 1.57	15.9 \pm 1.49	26.2 \pm 0.50	33.6 \pm 0.76
56 days	13.0 \pm 0.43	11.6 \pm 3.09	34.0 \pm 3.09	3.46 \pm 0.24	38.0 \pm 0.97
111 days	13.3 \pm 0.14	9.45 \pm 0.36	35.8 \pm 0.29	2.62 \pm 0.02	38.8 \pm 0.09
164 days	13.4 \pm 0.21	9.51 \pm 0.34	36.0 \pm 0.38	2.38 \pm 0.02	38.7 \pm 0.23
227 days	13.4 \pm 0.13	9.84 \pm 0.28	35.3 \pm 0.18	2.35 \pm 0.08	39.1 \pm 0.17
Maasdam cheese 2					
0 day	11.3 \pm 0.16	11.9 \pm 0.78	13.9 \pm 0.53	28.3 \pm 0.20	34.7 \pm 0.06
60 days	13.6 \pm 0.18	9.06 \pm 0.12	36.5 \pm 0.25	2.07 \pm 0.05	38.8 \pm 0.09
99 days	14.0 \pm 0.61	8.87 \pm 0.25	37.4 \pm 0.22	1.89 \pm 0.10	37.8 \pm 0.78
152 days	13.8 \pm 0.18	9.52 \pm 1.32	37.4 \pm 0.58	1.79 \pm 0.11	37.5 \pm 0.93
215 days	13.6 \pm 0.06	9.32 \pm 0.20	36.0 \pm 0.13	1.65 \pm 0.07	39.4 \pm 0.15

which had been hydrolysed by 56 days. The very rapid hydrolysis of α_{s1} -CN in the early phases of maturation coincides with the warm room ripening step between 10

and 56 days and reflects the observations by Lamichhane *et al.* (2018) who noted a significant increase in levels of primary proteolysis, that is from 5 to 17% pH 4.6-soluble

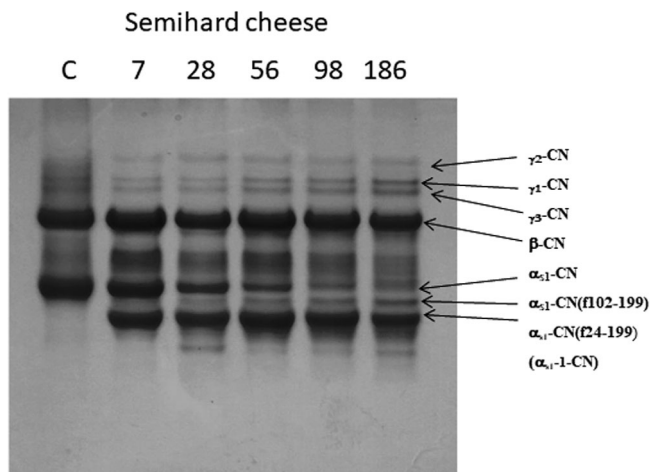


Figure 3 Urea-PAGE electrophoretogram of a semihard cheese at 7, 28, 56, 98 and 186 days of maturation. Lane C = sodium caseinate standard.

nitrogen as a percentage of total nitrogen (SN/TN) during this period.

The trends observed are in keeping with the use of chymosin as the coagulant, which would not be inactivated by the maximum scald temperatures used during the cheese manufacture (40 and 37 °C, respectively). Thus, the residual coagulant in the curd remained active during ripening similar to that reported by Sheehan *et al.* (2007). Some proteolysis may also arise from the activity of other enzymes such as cathepsin D. In addition, Maasdam cheese undergoes a warm room ripening phase (22 °C for 30- 40 days) early in maturation, thus accelerating the rate of α_{s1} -CN hydrolysis. This is contrary to Emmental maturation, where although a

similar hot ripening step is used, the coagulant is heat-labile and thus is inactivated during the higher scald temperatures (~50 °C) used during the cheese manufacture resulting in more intact α_{s1} -CN.

The small variance in the levels of the α_{s1} -CN observed between the two Maasdam-style cheeses most likely reflects slight batch-to-batch variations in the milk and thus cheese protein levels, make procedures, starter activities, etc. Reductions in α_{s1} -CN concentration levels during cheese maturation have also been noted by Soodam *et al.* (2015) who observed a 70% decrease in α_{s1} -CN levels in reduced-fat Cheddar cheese matured over 200 days.

Comparison of casein profiles observed between RP-HPLC and Urea-PAGE: β -CN

The RP-HPLC results only noted a minimal progressive degradation of β -CN in the semihard cheese and Maasdam cheeses over 186 days of maturation (Table 4) with an accumulation of γ -CNs. A greater accumulation of γ -CN 1 (β -CN f29–209) and γ -CN 3 (f108–209) was observed over maturation than γ -CN 2 (f106–209) by Urea-PAGE, however (Figures 3 and 4).

The curds were cooked to a max scald temperature of 40 °C during the cheese manufacture, which is slightly higher than for either Cheddar- or Gouda-type cheeses (usually 37–38 °C); however, given that thermophilic cultures were used as starters, the temperature is low in comparison with many Swiss- and Grana-type cheeses (usually 48–55 °C). Breakdown of β -CN during maturation is associated with plasmin activity, which in turn is more associated with high cook temperature cheeses. Increased rates of plasmin activity are linked to increased rate of plasminogen

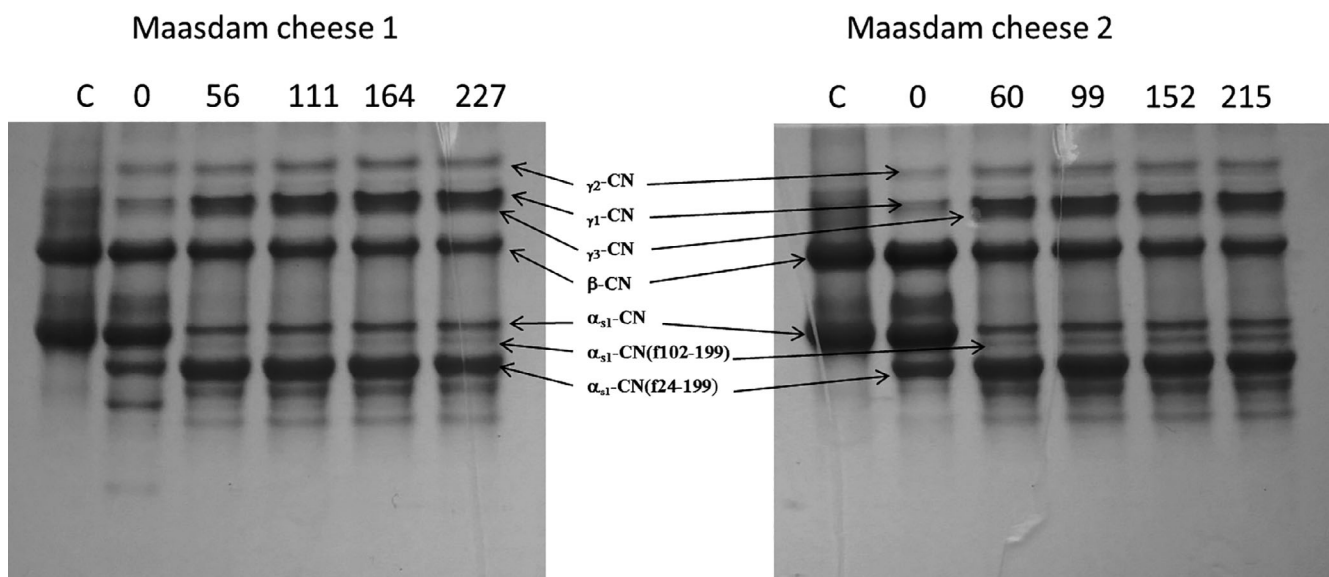


Figure 4 Urea-PAGE electrophoretograms of two Maasdam cheeses at 0, 56, 111, 164 and 227, or 0, 60, 99, 152 and 215 days of maturation. Lane C = sodium caseinate standard.

activation during maturation, possibly due to inhibitors of plasminogen activators and losses of plasmin to the whey during Cheesemaking (Farkye and Fox 1990) or to thermal inactivation of inhibitors of plasminogen activators. Somers *et al.* (2002) reported increased plasmin-induced proteolysis of β -CN to γ -CN in miniature cheeses cooked to 55 °C compared with those cooked at 48 °C.

CONCLUSIONS

The proposed RP-HPLC method offers the advantages of being simple and robust requiring just a standard HPLC with basic UV/Vis detection. The method can be easily used to track proteolysis and macromolecular changes that occur with the principal casein moieties; p- κ -, α_{s1} -, α_{s1} -I-, α_{s2} - and β -CN, during cheese maturation. In addition, the potential exists to concurrently monitor the progressive increase in small peptides and free amino acids in the insoluble portion of the same cheese extract by alternate pre-existing RP-HPLC or LC-MS methods. The main advantage of this method is that it is relatively inexpensive and easy to get accurate quantifiable information on all the major casein fractions in one step, especially for the insoluble p-k-casein. The authors believe that this method is applicable to all semihard and the majority of other cheese types, but further work is required to assess its suitability for soft cheeses or quarg.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the financial support of Dairy Levy Trust Fund (RMIS 6259) administered by Dairy Research Ireland. The authors would also like to acknowledge the assistance of the Technical Services Laboratory and Ms Juliet Wiley, Teagasc Food Research Centre, Moorepark, for cheese compositional analysis.

CONFLICT OF INTEREST

We have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Bernard Martin Corrigan: Conceptualization, data curation, formal analysis, investigation, methodology, project administration, resources, software, supervision, validation, visualization, writing-original draft, writing-review & editing. **Kieran Noel Kilcawley:** Investigation, resources, supervision, writing-review & editing. **Jeremiah J Sheehan:** Conceptualization, data curation, funding acquisition, resources, supervision, writing-original draft, writing-review & editing.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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