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Master Thesis

Examining the validity of a new rapid method for mapping out proteins in milk

Utvärdering av en ny tidseffektiv metod för profilering av mjölks proteinkomposition

By

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Abstract

Milk protein compositions play a pivotal role in physicochemical and technological properties of milk, particularly in rennet coagulation in cheese production. Conventional analytical methods such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography–high resolution mass spectrometry (LC-HRMS) provide detailed protein characterization but require long analysis times, extensive sample preparation and specialized instruments. Therefore, a need for another rapid method for practical routine protein profiling in dairy applications.

The aim of this study was to validate microfluidic chip electrophoresis using the Agilent 2100 Bioanalyzer and Protein 80 kit as a rapid method for milk protein profiling and to investigate if protein profiles obtained by this technique are correlated with rennet-induced coagulation properties. Raw milk samples collected from seventeen dairy farms and four dairy plant silos in southwestern Sweden were analysed. Protein profiles obtained by the Bioanalyzer were compared with LC-HRMS data, and relationships between protein fractions and rheological parameters were evaluated.

The Agilent 2100 Bioanalyzer successfully detected and profiled the major milk protein fractions, including α-casein (α-CN), β-casein (β-CN), κ-casein (κ-CN), β-lactoglobulin (β-LG), and α-lactalbumin (α-LA). Good reproducibility was observed for the major protein peak and overall protein distribution patterns showed agreement with LC-HRMS. However, differences were observed for certain protein fractions, particularly proteins with overlapping peaks and post-translational modifications.

Correlation plots revealed moderate relationships between casein fractions and rheological properties. Among the caseins, κ-CN showed the strongest association with yield stress, suggesting a potential contribution to gel network formation during rennet coagulation. Nevertheless, this indicated that milk coagulation behaviour is influenced by multiple factors beyond protein composition alone.

This study is managed within the PhD project “From farm to dairy: Sustainable milk quality for profitable cheese production” by Simon Höxter. By validating this rapid protein profiling technique and correlating its relationship to coagulation properties, this thesis contributes to the development of more efficient measurement for characterizing milk quality in industrial cheese production.

To summarize, microfluidic chip electrophoresis using the Agilent 2100 Bioanalyzer demonstrates potential as a rapid and reproducible method for milk protein profiling. Although further experiments are required before the method can be considered fully suitable for quantitative protein analysis, its speed, automation, and low sample requirements make it a promising screening tool for dairy research and industrial applications.

Table of Contents

1. Introduction

1.1. Overview

Cow milk proteins can be classified into two main groups of caseins and whey proteins which are structurally and fundamentally different (Fox et al., 2015). Caseins are synthesized in the mammary gland which represent approximately 80% of total milk protein. They are organized into large colloidal aggregates known as casein micelles, which are stabilized by colloidal calcium phosphate and protein–protein interactions (Fox et al., 2015). In contrast, whey proteins representing the other 20% are soluble, simple quaternary structure proteins dispersing in the aqueous phase of milk and are rich in sulphur. The structural and physicochemical differences are critical in cheese production and strongly influence how milk coagulates and how proteins are detected in milk systems (Fox et al., 2015).

Conventional methods for milk protein analysis include sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography–high resolution accurate mass spectrometry (LC-HRMS). These techniques provide detailed profiling of milk protein composition and genetic variants. However, these methods can be associated with limitations such as extensive sample preparation, long analysis times, low throughput, and the requirement for specialized instrumentation, depending on the analytical technique used (Miranda et al., 2020).

Despite the availability of advanced analytical methods, their complexity, cost, and long analysis times highlight the need for faster and simpler analytical alternatives. Microfluidic chip electrophoresis using the Agilent 2100 Bioanalyzer is a promising alternative, however it has insufficient validation on its ability to profile milk protein fractions in milk (Miranda et al., 2020; Sheng et al., 2022; Acquavia et al., 2025).

In addition to protein identification and quantification, understanding how protein composition influences coagulation properties remain an important area of dairy research. Variations in the relative abundance of individual milk proteins may affect gelation behaviour, curd formation, and cheese-making performance. However, the relationship between detailed protein profiles and rennet-induced coagulation properties is not yet fully understood and requires further investigation (Fox et al., 2017). The Agilent 2100 Bioanalyzer uses microfluidic electrophoresis combined with fluorescent detection to profile proteins according to their molecular weight. This system is highly automated and allows multiple samples simultaneously analysed with high reproducibility while requiring very small volumes of samples. The Agilent 2100 Bioanalyzer has previously been applied in various biological and food-related analyses, including protein profiling, nucleic acid analysis, and characterization of dairy proteins (Costa et al., 2014; Agilent Technologies, 2011). Its potential for rapid profiling makes it a promising tool for milk protein analysis. This will give a better insight into milk protein profiling and future work for technological properties of milk.

1.2. Aim

The aim of this study is to validate a new, rapid method using microfluidic chip electrophoresis in Agilent 2100 bioanalyzer with the Protein 80 kit for profiling milk protein. In addition, the study investigates whether protein profiles obtained by microfluidic chip electrophoresis are associated with rennet-induced coagulation

properties. Understanding these relationships may provide insight into the suitability of rapid protein profiling methods for dairy applications.

Main objectives are:

1. Validating a microfluidic chip electrophoresis method for characterizing the protein profile of milk.
2. To characterize protein profiles in skim milk and whey fractions using microfluidic chip electrophoresis and compare the results with LC-HRMS.
3. Compare milk protein profile data obtained by microfluidic chip electrophoresis with the result from LC-HRMS to assess method reliability.
4. Evaluate whether protein profiles obtained by microfluidic chip electrophoresis are associated with rennet-induced coagulation properties and can provide information relevant to milk coagulation behaviour.

2. Literature Background

2.1 Milk composition

Milk is a fluid secreted by the mammary glands of female mammals to provide complete nutrition to their newborns (Fox et al., 2015). Beyond its nutritional role, milk also serves several physiological functions for the newborn, mainly through bioactive proteins and peptides — including immunoglobulins, enzymes, growth factors, and antibacterial agents (Fox et al., 2015). Among milk-producing species, bovine milk is the most widely studied and serves as the primary raw material for the global dairy industry (Huppertz, 2025).

Bovine milk consists of approximately 87% water. The remaining solids are made up of fat, protein, lactose, and minerals (Tetra Pak, 2025), as summarized in Table 1. In addition to these major components, milk contains several hundred minor compounds, including vitamins, metal ions, and flavour compounds, many of which significantly contributed to the nutritional, technological, and sensory properties of milk and dairy products (Fox et al., 2015). This compositional complexity makes it difficult to fully analyse the composition of milk using a single analytical method (Acquavia et al., 2025).

Table 1. *Quantitative composition of bovine milk (Tetra Pak, 2025)*

Milk component	Typical range, g/100g	Average value, g/100g
Water	85.5-89.7	87.5
Fat	2.5-6.0	3.9
Proteins	2.9-5.0	3.4
Lactose	3.6-5.5	4.8

Based on its physicochemical organization, milk can be described as a complex polydisperse system

composed of three major phases: an aqueous phase known as milk serum, a colloidal phase containing casein micelles, and an emulsified fat phase (Fox et al., 2015). The milk serum contains lactose, salts, vitamins, and other small molecules (Farrell et al., 2004). In milk, proteins exist in two main forms: whey proteins remain dissolved as individual molecules in the milk serum, whereas most caseins formed large colloidal aggregates called casein micelles. Casein micelles range from 50 to 600 nm in diameter, but most are approximately 100-150 nm. Milk fat is dispersed as an emulsion, with fat globules ranging from 0.1 to 20 μm in diameter. Together, these structural features make milk a heterogenous polydisperse system, one in which particles of very different sizes coexist, contributing to the behaviour and analysis of milk proteins.

The composition of bovine milk varies considerably between cows. Key influencing factors include breed, stage of lactation, diet, health status (particularly mastitis), age, and milking intervals (Fox et al., 2015; Huppertz, 2025). Notably, variation is not limited to the amounts of each component — the chemical nature of certain constituents can also change. For example, the fatty acid profile of milk fat is strongly influenced by the cow's diet (Fox et al., 2015). In commercial bulk milk, much of this individual variation is averaged out, although notable variation may persist in regions where milk production is seasonal (Kelly & Bach Larsen, 2010).

Among all milk components, proteins are considered one of the most comprehensively important components due to their major contribution to both nutritional and physiological roles, including immune defence, enzymatic activity, and antimicrobial protection (Fox et al., 2015). Milk proteins account for approximately 3.0–3.5% of bovine milk and are commonly classified into two main groups: caseins and whey proteins (Huppertz, 2025). Detailed characterization of these proteins is therefore essential for understanding milk functionality and for developing analytical methods capable of evaluating protein composition and protein-related milk quality traits.

Because milk proteins exist in different physicochemical states, their distribution changes depending on the milk fraction analysed (Fox et al., 2015). Whey obtained after coagulation contains primarily soluble whey proteins, including β-lactoglobulin and α-lactalbumin, whereas most caseins are removed together with the coagulum (Walstra et al., 2006). In contrast, ultracentrifugation separates the milk serum phase from the colloidal casein micelle fraction without acid or enzymatic coagulation, allowing soluble proteins together with dissociated casein molecules to remain in the ultra-centrifugate phase (Farrell et al., 2004; Huppertz, 2025). Previous studies in Salunke et al., (2021) on milk fractionation and membrane separation have demonstrated that the partitioning of proteins between colloidal and serum phases depends on micellar stability and processing conditions. Therefore, differences are expected between whole milk, whey, and ultra-centrifugate protein profiles, particularly in the relative abundance of β-CN and whey proteins. These differences are important for understanding milk protein partitioning and for evaluating analytical methods used for protein profiling (Fox et al., 2015). Therefore, understanding the structure, properties, and distribution of milk proteins is essential for accurate protein characterization and interpretation of analytical protein profiles.

2.2. Milk proteins

2.2.1 Classification and nomenclature

Milk proteins were first classified into two distinct groups casein and whey by the Swedish scientist Hammarsten in the 1880s (Fox et al., 2015). He showed that when milk is acidified to pH 4.6 at approximately 30°C, around 80% of the total protein in bovine milk precipitates. This precipitate was defined as casein, while the proteins that remained soluble under these conditions were collectively called whey proteins (also referred to as serum proteins). Based on solubility at the isoelectric pH, this operational definition remains the standard basis for classifying milk proteins today (Farrell et al., 2004). In bovine milk, casein accounts for approximately 26 g/kg of milk and whey proteins approximately 6.3 g/kg, giving a casein-to-whey ratio of roughly 80:20 (Huppertz, 2025). By comparison, human milk has a casein-to-whey ratio of approximately 40:60, reflecting the different nutritional and physiological requirements of human infants (Fox et al., 2015).

Table 2 presents the average concentration, relative abundance, approximate molar mass, and selected physicochemical properties of the major bovine milk proteins, including their phosphorylation status, glycosylation, disulfide bond formation, and solubility at the isoelectric point (IEP) (Huppertz, 2025).

Table 2. *Proteins in Milk (From Huppertz, T. 2025)*

Protein	g/kg milk	g/100g protein	Molar mass (kDa)	Remarks
Casein	26	78.3	–	Isoelectric point (IEP) ≈4.6
αS1-CN	0.7	32	~23.6	Phosphoprotein
αS2-CN	2.8	8.4	~25.2	Phosphoprotein Contains -S-S- bridges Dimer
β-CN	8.6	26	~24.0	Phosphoprotein
κ-CN	3.1	9.3	~19.6	Glyco-phosphoprotein Contains Cysteine Oligomers
Whey Proteins	6.3	19	–	Soluble at IEP
β-Lactoglobulin	3.2	9.8	18.3	Contains -S-S- bridges Free -SH
α-Lactalbumin	1.2	3.7	14.2	Part of lactose synthase
Bovine serum albumin (BSA)	0.4	1.2	66.3	Blood protein
Proteose peptone	0.8	2.4	4-40	
Immunoglobulins	0.8	2.4	–	Glycoproteins
IgG, IgG2	0.65	1.8	∼150	
IgA	0.14	0.4	∼385	
IgM	0.05	0.2	∼900	
Miscellaneous	0.9	2.7	–	
Lactoferrin	0.1	–	86.0	Glycoproteins, bonds Fe

The nomenclature of bovine milk proteins follows the system established by Farrell et al. (2004), which represents the sixth revision of the official classification system. Under this system, caseins are designated by Greek letters reflecting their physicochemical properties: αs1-casein (α_{s1}-CN), α_{s2}-casein (α_{s2}-CN), β-casein (β-CN), and κ-casein (κ-CN). The subscript "s" in α_{s1}-CN and α_{s2}-CN indicates their sensitivity to calcium ions, as these proteins precipitate at physiological calcium concentrations. κ-CN, by contrast, is calcium-insensitive and plays a distinct stabilizing role in the casein micelle. A fourth casein, γ-casein (γ-CN), is also found in milk but is not considered an independent protein but rather as the fragments produced by the enzymatic degradation of β-CN. The degradation is caused by plasmin, which is the principal indigenous protease in milk. The major whey proteins are named individual, β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA), and the immunoglobulins (Ig).

Genetic variants within milk proteins are designated by capital letters appended to the protein name, such as β-CN A1/A2 and β-LG A/B (Farrell et al., 2004; Fox et al., 2015). When an animal is homozygous at the relevant locus, both alleles encode the same variant (e.g., β-LG AA or β-CN A2A2), whereas heterozygous animals express two different variants simultaneously (e.g., β-LG AB or β-CN A1A2). These genetic polymorphisms influence physicochemical and technological properties of milk, including protein composition, coagulation behaviour, and electrophoretic mobility (Fox et al., 2015). Variants within a protein typically differ by only one to three amino acid substitutions, yet these differences can affect the charge, hydrophobicity, and functional properties of the protein (Farrell et al., 2004). The genetic variants present in an individual animal's milk are determined by the alleles inherited at each locus, following codominant inheritance; both alleles are expressed, so a heterozygous animal produces two variants of a given protein simultaneously (Huppertz, 2025). The distribution of variants differs considerably between cattle breeds, contributing to the breed-associated differences in milk composition and processing behaviour (Poulsen et al., 2013). The variants present in a milk sample therefore reflect both the genetic background of the individual animal and the breed composition of the herd.

2.2.2 Physicochemical properties and post-translational modifications

The physicochemical properties of milk proteins are significantly influenced by post-translational modifications (PTMs), which alter protein structure after biosynthesis and have direct consequences for their behaviour during electrophoretic analysis (Fox et al., 2015; Huppertz, 2025). Three types of PTMs are relevant to the proteins discussed in this thesis: phosphorylation, glycosylation, and disulfide bond formation.

Phosphorylation is the most prevalent PTM among the caseins. It occurs at serine residues and, to a lesser extent, threonine residues. The degree of phosphorylation differs markedly between caseins: α_{s1}-CN carries 8 to 9 phosphate groups, α_{s2}-CN usually carries 10 to 13, β-CN carries 4 to 5, and κ-CN carries only 1 to 2 (Fox et al., 2015). These differences contribute to variation in apparent molecular mass between casein fractions during electrophoresis.

Glycosylation occurs exclusively on κ-CN, which carries O-linked oligosaccharide chains at multiple serine and threonine residues. Carbohydrate content accounts for approximately 5% of the molecular mass of κ-

CN, with individual molecules carrying between 0 and 4 oligosaccharide chains (Fox et al., 2015; Huppertz, 2025). Because the number of attached chains varies between molecules, κ-CN exists as a heterogeneous population with a broad molecular mass distribution (Fox et al., 2015; Huppertz, 2025). This heterogeneity explains why κ-CN typically appears as a broad, diffuse band or peak in electrophoretic separation profiles rather than a sharp, well-defined peak (Fox et al., 2015).

Disulfide bonds arise from oxidative cross-linking between cysteine residues. Among the caseins, α_{s1}-CN and β-CN contain no cysteine and therefore form no disulfide bonds, whereas α_{s2}-CN and κ-CN each contain two cysteine residues that can participate in intermolecular disulfide bond formation (Fox et al., 2015). Under non-reducing conditions, κ-CN in particular forms oligomers ranging from dimers to decamers, with apparent molecular masses far exceeding that of the monomer. Under reducing conditions, intermolecular disulfide bonds are cleaved, allowing proteins to migrate primarily as monomers during SDS-based electrophoretic separation (Fox et al., 2015; Anema, 2009).

The major proteins of bovine milk span a considerable range of molecular masses and differ substantially in their physicochemical properties, including isoelectric point, hydrophobicity, and degree of post-translational modifications. The four caseins (αs1-CN, αs2-CN, β-CN, and κ-CN) are relatively similar in monomeric molecular mass, ranging from approximately 19 to 25 kDa, but differ markedly in phosphorylation level, glycosylation, hydrophobicity, and disulfide bond formation (Fox et al., 2015). Among the caseins, β-CN exhibits particularly high hydrophobicity, whereas κ-CN is distinguished by glycosylation and its ability to form intermolecular disulfide-linked oligomers. The major whey proteins cover a broader molecular mass range. β-Lactoglobulin (β-LG) has a monomeric molecular mass of approximately 18 kDa but exists predominantly as a homodimer under native conditions at neutral pH. α-Lactalbumin (α-LA) has a molecular mass of approximately 14 kDa, whereas bovine serum albumin (BSA) has a molecular mass of approximately 67 kDa. The immunoglobulins are considerably larger proteins, with intact IgG having a molecular mass of approximately 150–160 kDa (Fox et al., 2015; Huppertz, 2025). Table 3 summarizes selected structural and physicochemical properties of the major bovine milk proteins, including molecular mass, phosphorylation, cysteine content, glycosylation, hydrophobicity, isoelectric point, and genetic variants.

Table 3. *Properties of Some Milk Proteins (From Huppertz, T. 2025)*

Protein	Molar mass	Amino acid	Phosphoserine	Cysteine / –S–S- linkages	Hexoses	Hydrophobicity
Unit		res./mol	res./mol.	Res./mol. /mol.	Res./mol.	%
α_{s1}-CN (B)	23.614	199	8	0/0	0	25
α_{s2}-CN (A)	25.230	207	11	2/1	0	23
β-CN (A^2)	23.983	209	5	0/0	0	29
κ-CN (A)	19.023[a]	169	1	2/-[b]	∼2.3[c]	22
β-LG (B)	18.283	162	0	5/2	0	29
α-LA (B)	14.176	123	0	8/4	0	28

[a] *Exclusive of carbohydrate residues.*

[b] *κ-CN preferentially forms intermolecular rather than intramolecular disulfide bonds, resulting in polymeric structures with a variable number of S–S linkages per molecule.*

[c] *Average.*

[d] *% hydrophobic side groups (Val, Leu, Ile, Phe, Trp).*

2.2.3. Structural properties of bovine caseins

The caseins share several structural features that distinguish them from globular proteins. Due to their high proline content, which disrupts the formation of α-helices and β-sheets, the caseins adopt open, flexible conformations with little defined secondary or tertiary structure. This structural openness makes them highly susceptible to proteolysis but also confers exceptional heat stability; caseins do not denature below 100°C, unlike the whey proteins (Fox et al., 2015). The major bovine caseins differ considerably in their amino acid composition and structural organization. αs1-CN and αs2-CN contain large clusters of phosphorylated residues that contribute to calcium binding and micelle formation. β-CN contains a high proportion of hydrophobic amino acids, giving it amphiphilic properties and promoting its temperature-dependent dissociation from the casein micelle. In contrast, κ-CN contains cysteine residues capable of forming disulfide bonds and is the only casein fraction that can be glycosylated. Following chymosin cleavage during rennet coagulation, κ-CN loses its stabilizing function at the micelle surface, initiating micelle aggregation and gel formation (Fox et al., 2015).

κ-CN differs structurally from the other caseins because it contains cysteine residues capable of forming intermolecular disulfide bonds. In addition, a proportion of κ-CN molecules are glycosylated with O-linked oligosaccharides, resulting in considerable molecular heterogeneity within the κ-CN fraction (Fox et al.,

2015; Huppertz, 2025). The heterogeneous degree of glycosylation contributes to structural heterogeneity and influences the surface properties of κ-CN (Fox et al., 2015). This charge and hydrophobicity distribution gives β-CN surfactant-like properties. A notable feature of β-CN is its temperature-sensitive association with the casein micelle. At refrigeration temperatures (around 4°C), a substantial proportion of β-CN — estimated at approximately 10 to 50% — dissociates from the micelle and enters the serum phase, a process that is reversible upon warming (Fox et al., 2015; Huppertz, 2025). The high hydrophobicity of β-CN also affects its behaviour during SDS-PAGE and chip electrophoresis. Because β-CN binds more SDS per unit mass than the other caseins, it acquires a greater negative charge relative to its true molecular mass, causing it to migrate faster than expected. As a result, the apparent molecular mass of β-CN in denaturing electrophoresis is lower than its true value, and care must be taken when interpreting its position in an electrophoretic profile (Fox et al., 2015).

2.2.4 Whey proteins

Whey proteins are globular proteins with relatively compact and well-defined secondary and tertiary structures stabilized by intramolecular disulfide bonds. In contrast to the caseins, whey proteins are heat-sensitive and undergo irreversible denaturation at temperatures above approximately 70–80°C (Fox et al., 2015). The major whey proteins in bovine milk are β-lactoglobulin (β-LG) and α-lactalbumin (α-LA), while bovine serum albumin (BSA), immunoglobulins (Ig), lactoferrin, and several minor proteins are present at lower concentrations (Huppertz, 2025).

β-LG is the most abundant whey protein in bovine milk and is absent from human milk. The protein has a monomeric molecular mass of approximately 18 kDa but predominantly exists as a homodimer under native conditions at neutral pH (Fox et al., 2015). β-LG contains two intramolecular disulfide bonds and one free thiol (–SH) group. Upon heat denaturation, the protein unfolds and exposes the reactive thiol group, enabling thiol–disulfide exchange reactions with cysteine residues of κ-CN on the casein micelle surface. Formation of β-LG–κ-CN complexes influence the functional properties of heated milk systems and is particularly important during dairy processing operations involving thermal treatment (Fox et al., 2015).

Three major genetic variants of β-LG have been identified: A, B, and C. These variants differ by one or two amino acid substitutions and exhibit differences in conformational stability and dimerization behaviour (Huppertz, 2025). Variants A and B are the most common in bovine milk and may influence processing characteristics and electrophoretic mobility (Heck et al., 2009).

α-LA is the second most abundant whey protein in bovine milk, with a molecular mass of approximately 14 kDa. Unlike β-LG, α-LA contains no free thiol group, as all eight cysteine residues participate in four intramolecular disulfide bonds (Fox et al., 2015). A characteristic structural feature of α-LA is its calcium-binding site, which contributes significantly to stabilization of the native protein conformation. Biologically, α-LA functions as a regulatory subunit of lactose synthase, modifying galactosyltransferase activity during lactose biosynthesis in the mammary gland (Huppertz, 2025).

Bovine serum albumin (BSA) is a blood-derived whey protein with a molecular mass of approximately 67 kDa. Although present at relatively low concentrations in milk, BSA contributes to the overall whey

protein fraction and participates readily in heat-induced interactions due to its multiple disulfide bonds and reactive thiol groups (Fox et al., 2015). Immunoglobulins represent another group of minor whey proteins and include IgG, IgA, and IgM. IgG is the predominant immunoglobulin in bovine milk, whereas colostrum contains substantially higher immunoglobulin concentrations that decline rapidly after parturition (Huppertz, 2025). These proteins contribute to passive immune protection in the newborn calf.

2.2.5. Minor proteins and indigenous enzymes

In addition to the major casein and whey protein fractions, bovine milk contains several minor protein components. The proteose-peptone fraction consists primarily of peptides derived from the plasmin-mediated hydrolysis of β-CN, with the principal products designated γ-CN (γ1-, γ2-, and γ3-CN) and proteose-peptone components (PP5, PP8 fast, and PP8 slow). Although present in relatively small amounts, these fragments contribute to the heterogeneity observed in electrophoretic profiles of milk proteins (Fox et al., 2015).

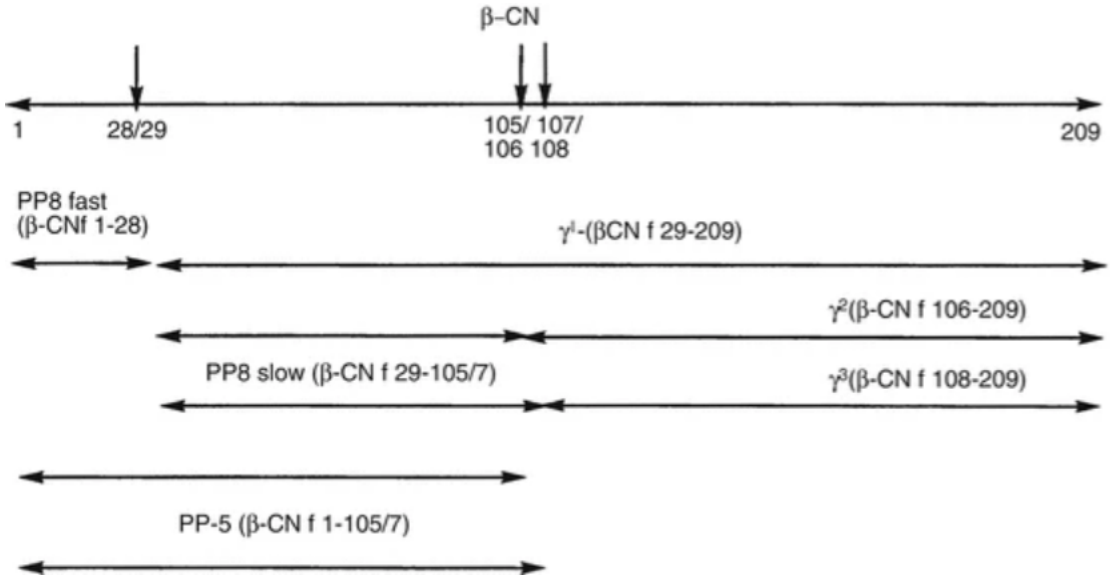


Fig. 1. *Principal products produced from β-CN by plasmin. From Fox et al., 2015.*

Lactoferrin is an iron-binding glycoprotein with antimicrobial properties, present at a molecular mass of approximately 80 kDa; it is found at relatively low concentrations in bovine milk but at considerably higher levels in human milk and bovine colostrum.

Finally, bovine milk contains a range of indigenous enzymes, including plasmin, lipoprotein lipase, and alkaline phosphatase, among others. Of relevance to this thesis is chymosin (rennet), a protease with a molecular mass of approximately 36 kDa that is responsible for the primary cleavage of κ-CN during cheese manufacture (Fox et al., 2015; Anema, 2009). Under reducing electrophoretic conditions of the Protein 80 kit, chymosin is expected to migrate in the low molecular mass region of the electrophoretic separation profile.

2.3. Casein micelle structure and rennet coagulation

Caseins are the predominant proteins in bovine milk and exist primarily as large colloidal aggregates known as casein micelles, with diameters typically ranging from 50 to 500 nm and an average size of approximately 150 nm (Fox and Brodkorb, 2008). The micelles are composed of α_{s1}-, α_{s2}-, β-, and κ-CN together with colloidal calcium phosphate (CCP), which plays an essential structural role in micelle organization and stability. Calcium-sensitive caseins, particularly α_{s1}-, α_{s2}-, and β-CN, interact with CCP through phosphoserine clusters, forming an internal protein-mineral network that stabilizes the micellar structure (Horne, 2020).

At the micelle surface, κ-CN provides steric stabilization through its hydrophilic C-terminal region, the casein macropeptide (CMP), which extends into the surrounding serum phase and forms a so-called "hairy layer" (Fox et al., 2015). This surface layer generates steric and electrostatic repulsion between micelles, preventing aggregation under normal milk conditions. A schematic illustration of the casein micelle structure is shown in Figure 2. Although several models of micelle organization have been proposed, current understanding generally agrees on the central structural role of CCP and the stabilizing function of surface-localized κ-CN (Dalgleish, 2011; Horne, 2020).

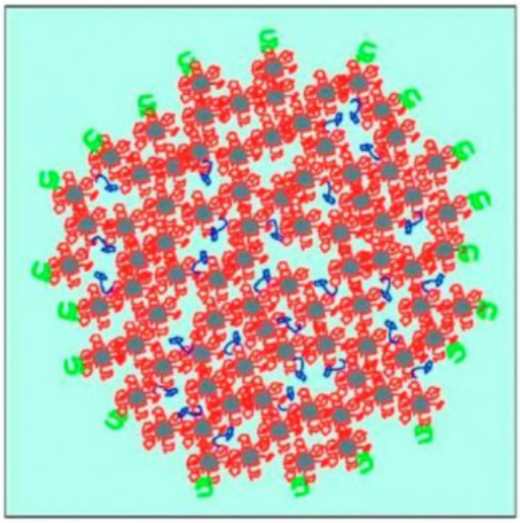


Figure 2. *Schematic illustration of the casein micelle structure, showing incorporated calcium phosphate nanoclusters with associated caseins and surface-localized κ-CN (adapted from Dalgleish, 2011).*

Rennet-induced coagulation, which forms the basis of most cheese manufacture, occurs in two principal stages. In the primary enzymatic phase, chymosin specifically hydrolyzes κ-CN at the Phe105–Met106 bond, releasing the hydrophilic CMP fragment into the surrounding serum and leaving para-κ-CN attached to the micelle surface (Calvo et al., 1995). Removal of the protective κ-CN layer destabilizes the micelles by eliminating the steric barrier that normally prevents close micelle-micelle interactions (Gregersen et al., 2016; Nilsson et al., 2020).

In the secondary aggregation phase, destabilized para-casein micelles aggregate in the presence of calcium ions to form a three-dimensional gel network that entraps fat globules and serum, producing the cheese curd.

The rate and extent of coagulation are influenced by several factors, including temperature, pH, ionic calcium concentration, and the composition of the casein fraction (Fox et al., 2015). In particular, the relative proportion of κ-CN strongly influences micelle size and coagulation behaviour. Milk containing higher κ-CN levels generally forms smaller micelles with larger surface area and improved rennet coagulation properties (Horne, 2020).

Variations in the relative abundance of αs1-, αs2-, β-, and κ-CN also influence micellar organization and the mechanical properties of the resulting curd. Consequently, characterization of casein composition is important for understanding variation in milk coagulation properties and cheese-making performance across different milk sources and processing conditions.

2.5. Conventional methods for milk protein analysis

2.5.1. Purpose of milk protein analysis

The purpose of milk protein analysis is to obtain information on the composition, distribution, and structure of proteins in milk. Variations in milk protein composition can influence the technological properties of milk, particularly coagulation ability and cheese-making performance (Fox et al., 2015). Therefore, characterization of milk proteins is important for understanding both the functional properties of milk and its suitability for dairy processing applications.

Typically, this includes the identification of different proteins, determination of their molecular weight, and their relative abundance in the milk sample. These analyses are important because changes in protein composition may occur due to genetic variation, post-translational modifications, heat treatment, proteolysis, or processing conditions (Walstra et al., 2006). Molecular weight is important for distinguishing different protein compositions while relative abundance provides details of the distribution of different proteins in a milk sample. Protein analysis is important in the dairy industry because protein composition influences the nutritional, functional, and technological properties of milk and dairy products, particularly in cheese manufacture and processing applications (Walstra et al., 2006; Fox et al., 2015).

2.5.2 Overview of analytical methods for milk protein analysis

Several analytical methods have been developed for milk protein analysis, and some are mainly used for total protein content, whereas some provide detailed profiling of individual protein components, isoforms and post-translational modifications.

Methods such as the Kjeldahl method are commonly used for total protein determination. Kjeldahl analysis estimates total nitrogen content which can be used to calculate total protein, total casein or total whey protein concentrations. However, the method provides limited information regarding individual protein fractions (Smith, 2017). Immunological methods such as enzyme-linked immunosorbent assay (ELISA) and Western blotting are used for targeted detection and quantification of specific milk proteins but require the availability of specific antibodies and is considered a time-consuming method, as the possibility of multiplex assays is restricted (Asensio et al., 2008).

Several analytical methods are available for milk protein analysis, including electrophoretic and

chromatographic techniques. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins primarily according to molecular weight, whereas chromatographic techniques such as reversed-phase high-performance liquid chromatography (RP-HPLC) separate proteins based on hydrophobic interactions (Smith, 2017). Two-dimensional gel electrophoresis combines separation by isoelectric point and molecular weight, enabling improved resolution of complex protein mixtures (Turner et al., 2006). More advanced methods, including capillary electrophoresis–mass spectrometry (CE-MS), imaged capillary isoelectric focusing (icIEF), and liquid chromatography–high-resolution mass spectrometry (LC-HRMS), allow detailed characterization of protein variants, isoforms, and post-translational modifications (Miranda et al., 2020; Sheng et al., 2022).

2.5.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis (PAGE) with sodium dodecyl sulfate (SDS) is widely used for the separation of protein subunits according to their molecular size. In SDS-PAGE, proteins are dissociated in a buffer containing SDS and a reducing agent before electrophoretic separation. SDS binds along the polypeptide chain, causing proteins to unfold and acquire a nearly uniform negative charge proportional to their mass. As a result, proteins are separated primarily according to molecular size rather than their native charge or conformation (Smith, 2017).

Reducing agents such as dithiothreitol (DTT) or β-mercaptoethanol are commonly included to cleave intermolecular and intramolecular disulfide bonds, ensuring that proteins migrate predominantly as moneric subunits during electrophoresis (Anema, 2009). Following electrophoretic separation through the polyacrylamide gel matrix, protein bands can be visualized and used to identify the major milk proteins, including αs-CN, β-CN, κ-CN, β-LG, α-LA, as well as proteolysis products derived from casein degradation (Di Marzo et al., 2021).

SDS-PAGE has been widely applied in dairy science to characterize milk protein composition and to distinguish protein patterns between different milk species and cattle breeds (Di Marzo et al., 2021). The technique is also widely used to evaluate heat-induced protein denaturation, proteolysis, and changes associated with milk processing. However, separation of the major caseins remains challenging because their molecular masses are relatively similar. In addition, β-CN exhibits unusually high electrophoretic mobility due to its high hydrophobicity and increased SDS binding, causing it to migrate faster than expected from its true molecular mass (Fox et al., 2015). Consequently, interpretation of casein band patterns in SDS-PAGE requires consideration of both molecular mass and physicochemical behaviour.

2.5.4. Liquid Chromatography - High Resolution Accurate Mass Spectrometry (LC-HRMS)

Liquid chromatography–high resolution mass spectrometry (LC-HRMS) combines chromatographic separation with mass spectrometric detection, enabling detailed characterization of complex milk protein mixtures (Miranda et al., 2020). Mass spectrometry parameters to identify protein are represented by mass-to-charge values and deconvolution algorithms to detect protein masses from the charge state distributions, which is used to identify e.g. β-CN genetic variants A1 with A2, glycosylated κ-CN, phosphorylated

isoforms and proteolysis products (Miranda et al., 2020).

Prior to mass spectrometric detection, milk proteins are chromatographically separated according to their physicochemical properties. According to Sheng et al., 2022, milk proteins are highly heterogeneous and exist as multiple genetic variants and post-translationally modified isoforms. Phosphorylation and glycosylation increase the molecular diversity of caseins, particularly κ-CN, making accurate characterization difficult using conventional analytical techniques. LC-HRMS provides high mass accuracy and resolution, allowing detection and quantification of intact proteins together with their isoforms and post-translational modifications more effectively than traditional protein separation methods (Miranda et al., 2020). Despite its strong analytical performance, LC-HRMS requires advanced instrumentation, long analysis times and complex data interpretation. Therefore, although LC-HRMS is a highly suitable reference method, simpler and faster profiling methods remain desirable for routine dairy applications.

2.6. Microfluidic chip electrophoresis using the Agilent 2100 Bioanalyzer and Protein 80 kit

Microfluidic chip electrophoresis is a downsized electrophoretic technique developed to perform rapid protein separation using integrated microfluidic channels fabricated within disposable chips (Bousse et al., 2001). The technique combines principles of SDS-PAGE with automated microfluidic technology, allowing protein separation, detection, and data processing to occur within a single chip. Compared with traditional gel electrophoresis methods, microfluidic systems require significantly smaller sample volumes, shorter analysis times, and reduced manual handling (Anema, 2009).

The Agilent 2100 Bioanalyzer together with the Protein 80 kit was used for rapid profiling of bovine milk proteins. The Protein 80 kit is designed for separation of proteins within a molecular weight range appropriate for major milk proteins, including caseins and whey proteins. Similar to SDS-PAGE, proteins are first denatured using SDS and reducing agents prior to electrophoretic separation, SDS then binds to proteins, enabling separation primarily according to molecular size (Smith, 2017). Reducing agent DTT assists in disruption of disulfide bonds and unfolding of protein structures during denaturation (Anema, 2009).

Within the microfluidic chip, proteins migrate through interconnected microchannels under an applied electric field and are separated according to their electrophoretic mobility (Wu et al., 2008). Following separation, proteins are detected by laser-induced fluorescence after interaction with fluorescent dye molecules present in the assay reagents. The Agilent 2100 Bioanalyzer software automatically generates electropherograms and virtual gel-like images for each sample. Protein molecular weights are estimated by comparison with a protein ladder included in the kit (Agilent Technologies, 2011). Compared with conventional SDS-PAGE, microfluidic chip electrophoresis integrates multiple analytical steps into an automated chip-based platform, reducing analysis time and sample handling requirements (Bousse et al., 2001).

Milk proteins represent a particularly complex analytical system because bovine milk contains several

casein fractions and whey proteins with relatively similar molecular masses. In addition, many milk proteins have several genetic variants and post-translationally modified isoforms which include phosphorylated and glycosylated forms (Fox et al., 2015). These molecular variations may influence electrophoretic migration behaviour and complicate separation of individual protein fractions. Several studies have demonstrated the applicability of microfluidic chip electrophoresis for milk protein characterization. Anema (2009) demonstrated that "lab-on-a-chip" microfluidic SDS electrophoresis could successfully separate major bovine milk proteins including α_s-CN, β-CN, κ-CN, β-LG, and α-LA. The method also showed potential for evaluating protein changes associated with heat treatment and proteolysis. Freire Costa et al. (2014) further investigated the influence of buffer systems and quantitative performance in microfluidic chip electrophoresis for milk protein analysis and reported good analytical reproducibility for several major milk protein fractions.

An important advantage of microfluidic chip electrophoresis is the rapid analytical time. According to the Protein 80 kit specifications, up to ten samples may be analysed simultaneously on a single chip with an approximate runtime of 30 minutes per chip (Agilent Technologies, 2011). The method also requires only microliter-scale sample volumes, thereby reducing reagent consumption and sample preparation requirements. These characteristics make the method attractive for industrial applications where large numbers of samples require rapid analysis. Despite these advantages, microfluidic chip electrophoresis provides lower molecular specificity and analytical resolution compared with advanced mass spectrometry-based methods such as LC-HRMS. LC-HRMS enables more detailed characterization of protein isoforms, genetic variants, post-translational modifications, and proteolysis products due to its high mass accuracy and molecular specificity (Miranda et al., 2020; Sheng et al., 2022). In addition, LC-HRMS analysis typically requires longer analysis times and more extensive sample preparation compared with microfluidic chip electrophoresis. In contrast, proteins with similar molecular masses may exhibit overlapping migration behaviour in microfluidic electropherograms, which can limit peak resolution and protein identification.

Nevertheless, the simplicity, speed, and automation of microfluidic chip electrophoresis make the method attractive for rapid routine protein profiling in dairy applications. In the present study, the Agilent 2100 Bioanalyzer together with the Protein 80 kit was evaluated as a potential rapid alternative for bovine milk protein profiling and compared with LC-HRMS as the reference analytical method.

2.7. Validation of the rapid protein profiling method

Validation of rapid analytical methods is essential before implementation in industrial dairy applications and research laboratories. Although advanced analytical techniques provide highly detailed characterization of milk proteins, these methods often come with expensive instrumentation, complex data analysis, and long analysis times. Consequently, there is increasing interest in developing simpler and faster analytical methods capable of providing sufficiently reliable protein profiling information for routine applications (Miranda et al., 2020).

Validation of the method focused on determining whether microfluidic chip electrophoresis could provide

protein profiling results comparable to those obtained using LC-HRMS despite shorter analysis time and simplified sample preparation. Attention has been mainly given to the major milk protein fractions, especially α-CN, β-CN, and κ-CN, because these proteins are strongly associated with coagulation behaviour and cheese-making properties for rheological comparisons (Fox et al., 2015).

Milk proteins are highly heterogeneous and occur as multiple phosphorylated and glycosylated isoforms. This molecular complexity makes detailed protein characterization analytically challenging (Sheng et al., 2022). LC-HRMS can distinguish many of these protein variants due to its high resolution and accurate mass determination, whereas electrophoretic methods primarily separate proteins according to migration behaviour and apparent molecular weight. Therefore, direct comparison between the two techniques provides important information regarding the analytical capability and limitations of microfluidic chip electrophoresis (Miranda et al., 2020; Sheng et al., 2022; Acquavia et al., 2025).

Evaluating reproducibility is important when validating a method. For rapid screening methods to become viable in industrial environments, the analytical results must remain stable between repeated analyses. In microfluidic electrophoresis, several factors may influence reproducibility, including protein denaturation efficiency, chip loading consistency, and variability in electrophoretic migration. Overlapping peaks may occur because several milk protein fractions possess similar molecular masses (Sheng et al., 2022). Another important aspect is peak resolution. In particular, α-CN and κ-CN regions may contain overlapping signals generated from multiple isoforms and post-translationally modified proteins. Consequently, interpretation of electropherograms may become more difficult compared with LC-HRMS datasets, where chromatographic separation and high-resolution mass analysis provide improved protein identification (Miranda et al., 2020).

Rheological measurements are widely used to evaluate the development and mechanical properties of milk gels during rennet coagulation. Parameters such as gelation time (tg), storage modulus (G′), and yield stress provide information regarding the formation, firmness, and structural stability of the casein gel network (Fox et al., 2017; Tunick, 2013). Variations in milk protein composition, particularly the relative proportions of casein fractions and mineral balance, may influence coagulation of kinetics and gel structure formation. For example, differences in κ-casein content have been associated with changes in micelle stability, curd formation, and gel firmness during rennet coagulation (Fox et al., 2015). Consequently, rheological analysis is commonly applied in dairy research to investigate the relationship between milk protein composition and coagulation behaviour, as well as to assess factors affecting cheese-making properties and dairy processing performance.

2.8. Rheological properties

Rheological measurements are used to characterize the flow and deformation behaviour of semi-solid food materials (Zhong and Daubert, 2013). Most semi-solid materials are viscoelastic materials whose properties cannot be simply differentiated based on viscosity alone. For Newtonian fluids, μ is the Newtonian viscosity describing the proportionality between shear stress and shear strain rate. For a pure viscous material, the mechanical energy applied to this material is converted to other forms of energy and cannot be recovered

after deformation (Zhong and Daubert, 2013). Calculation of viscosity (μ) is given below (Tharayil et al., 2025). Rheological parameters therefore provide useful information regarding the structural development and mechanical properties of milk samples.

The gelation process, generally referred to as the secondary phase of rennet coagulation, initially involves the formation of chains and clusters of micelles, eventually leading to the formation of a continuous gel network (Fox et al., 2017). Gelation time (t_g) is commonly defined as the point at which a measurable gel network begins to form after coagulation initiation, although different rheological criteria have been used in the literature (Lucey, 2002). Differences in gelation time may reflect variations in milk composition, casein micelle properties, mineral balance, and coagulation behaviour. In general, a shorter t_g indicates earlier onset of gel formation.

Young's modulus or the modulus of elasticity is the ratio of stress to strain during compression and indicates the stiffness of the sample at fracture (Tunick, 2013; Lucey, 2002). A curve of force versus strain may show an inflection point where the structure begins to break down. Eventually the structure collapses faster than the stress build-up; the stress at this fracture point is the yield stress (Tunick, 2013). Higher yield stress values indicate greater resistance of the gel network to mechanical breakdown.

Storage modulus (G') characterizing the food matrix was measured in the linear viscoelastic region of strain (Mohan et al., 2022), it represents the elasticity of the sample. Stress sweeps for controlled stress rheometers were calculated and are used to locate the linear viscoelastic region (LVR) when controlled stress tests are made (Tunick, 2013). The formulae for calculations of G' is given below (Tharayil et al., 2025).

Calculation of G'

$$G' = \frac{\sigma_{amplitude}}{\gamma_{amplitude}} cos(\delta)$$

Where $\sigma_{amplitude}$ is the stress amplitude, $\gamma_{amplitude}$ is the strain amplitude and δ is the phase difference between stress and strain.

3. Materials and Methods

3.1 Milk collection, storage and sample preparation

Raw milk samples were collected from seventeen dairy farms and four dairy plant silos distributed between two dairy plants in southwestern Sweden during February and March 2025. The geographical area ranged from 57°8′ to 58°6′ N and 13°0′ to 14°0′ E. Following the collection, the milk samples were transported to the laboratory under refrigerated conditions. Rheological measurements were performed on fresh milk samples prior to freezing. Milk samples used for protein analysis were stored at -80°C within 48 hours of collection. LC-HRMS analysis was performed on frozen milk samples at the Arla Innovation Centre (Aarhus, Denmark) in May 2025.

Commercially available organic non-standardized milk (Arla Ko® Ekologisk Färsk Lantmjölk; Arla Foods, Sweden; 3.8 to 4.5% fat, low-pasteurized and non-homogenized) purchased from local supermarket in Lund,

Sweden, and was additionally used for whey and milk serum preparation.

3.1.1. Preparation of skim milk

Skim milk was prepared from both farm milk and commercial whole milk by centrifugation. Milk samples (approximately 400–600 g) were centrifuged at 2,600 × g for 30 minutes at 4°C using an Allegra X-15R centrifuge (Beckman Coulter, USA). After centrifugation, the fat layer was carefully removed, and the skim milk fraction was collected without touching the residual fat as much as possible. Prior to Bioanalyzer analysis, skim milk samples were diluted 1:10 (v/v) with deionized water and prepared according to the denaturation procedure described in Section 3.3.

3.1.2. Preparation of sweet whey

Sweet whey was prepared from commercial whole milk by rennet induced coagulation. The native pH of the milk was recorded and adjusted to pH 6.50 ± 0.02 using lactic acid. Milk samples were tempered at 32°C for 30 minutes in a water bath before rennet (Chy-MAX® Plus, Chr. Hansen, Denmark) was added to a final concentration of 0.09 IMCU/mL milk. The samples were briefly mixed and incubated at 32°C for 40 minutes to allow coagulation.

Following coagulation, the curd was manually disrupted, and the whey fraction was separated using a sieve. Whey samples were cooled at 4°C for approximately 60 minutes and subsequently stored at -80°C until analysis.

Initial Bioanalyzer analysis of undiluted whey samples resulted in signal intensities exceeding the linear detection range of the instrument. Therefore, whey samples were further diluted 1:5 and 1:2 (v/v) with deionized water before analysis according to the denaturation procedure described in Section 3.3.

3.2. Reagents and sample preparation for microfluidic chip electrophoresis

Reagent preparation and chip preparation were performed according to the Agilent Protein 80 Kit Guide (Agilent Technologies, Waldbronn, Germany). The Agilent 2100 Bioanalyzer system together with the Agilent Protein 80 kit was used for protein analysis. The kit included Protein 80 chips, Protein 80 gel-matrix, dye concentrate, sample buffer and Protein 80 ladder. Chip preparation was performed using the chip priming station supplied with the Bioanalyzer system. Sample preparation and analysis were carried out using micropipettes with sterile pipette tips, 0.5 mL microcentrifuge tubes, and microcentrifuge (Eppendorf AG, Hamburg, Germany). Protein denaturation was performed using a water bath. Deionized water was used for dilution and sample preparation, while 1 M dithiothreitol (DTT; Sigma-Aldrich, St. Louis, MO, USA) solution was used as a reducing agent during denaturation.

Protein 80 dye concentrate and gel-matrix were allowed to equilibrate to room temperature for 30 min. The content of Protein 80 gel-matrix vial (650 µL) was transferred into a spin filter and centrifuged at approximately 2,500 × g for 15 minutes. Subsequently, 25 µL of dye concentrate was added to the filtered gel and vortexed for 10 to 20 seconds until a uniform colour was obtained. The destaining solution (DS) was prepared by transferring 650 µL of Protein 80 gel-matrix into a spin filter followed by centrifugation at 2,500 x g for 15 minutes. The filtered solution was collected and labelled as DS during chip preparation to reduce the background fluorescence and improve electrophoretic separation quality (Agilent Technologies,

2011). The denaturing solution was prepared by adding 3.5% (v/v) of 1 M DTT solution to the Protein 80 sample buffer. The mixture was vortexed for 5 seconds to ensure complete mixing. The denaturing solution was used to reduce disulfide bonds in proteins during sample preparation prior to electrophoretic separation.

3.3. Preparation of sample and protein ladder

4 µL of milk protein samples were mixed with 2 µL of denaturing solution in 0.5 mL microcentrifuge tubes (Eppendorf®, Hamburg, Germany) Samples and 6 µL aliquots of Protein 80 ladder were heated at 95°C for 5 minutes in a water bath and subsequently cooled to room temperature. The samples were mixed using a vortex mixer for 15 seconds before addition of 84 µL deionized water, followed by another brief vortex-mixing to ensure homogeneous mixing prior to analysis.

3.4. Chip preparation and sample loading

Prior to chip preparation, the gel-dye mixture was allowed to equilibrate to room temperature for 30 minutes while protected from light. A new Protein chip was placed on the chip priming station and 12 µL of gel-dye mixture was pipetted into the well-marked "G". The chip was pressurized using the chip priming station according to the Protein 80 Kit Guide (Agilent Technologies, 2011) for 60 seconds to ensure proper distribution of the gel matrix within the microchannels.

After priming, 12 µL of gel–dye mixture was pipetted into all wells labelled "G" and 12 µL of destaining solution was added to the well labelled DS. Care was taken to pipette the gel–dye mixture at the bottom of the well to prevent the formation of air bubbles. 6 µL of each prepared milk sample was pipetted into the sample wells, then 6 µL of the Protein 80 ladder was pipetted into the well-marked with the ladder symbol. All wells were filled to ensure proper chip operation during microfluidic chip electrophoresis. The prepared chip was subsequently inserted into the Agilent 2100 Bioanalyzer for analysis. An overview of how the wells are filled can be found in Figure 3.

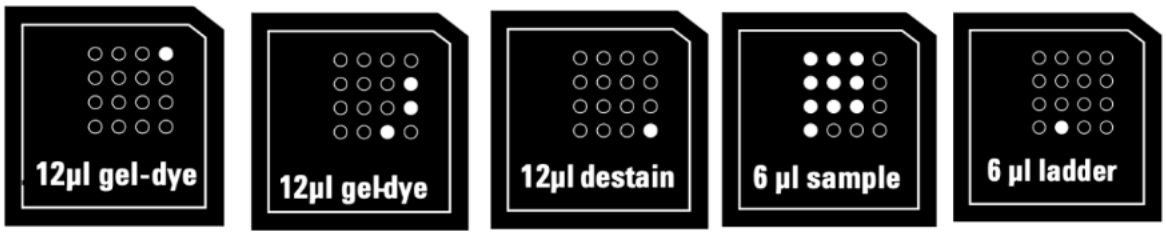


Figure 3. *Overview of the Agilent 2100 Bioanalyzer Protein 80 chip preparation procedure from Agilent Protein 80 Kit Guide (Agilent Technologies, 2011).*

3.5. Bioanalyzer analysis

The chip was inserted into the Agilent 2100 Bioanalyzer, and the Protein 80 assay kit was selected on the 2100 Expert software. Each chip allowed analysis of up to 10 samples with a total run time of approximately 30 minutes per chip. Protein separation was carried out according to the manufacturer's kit guide. The software automatically generated electropherograms and gel-like images for each sample, while molecular weights of the detected protein peaks were estimated using the Protein 80 ladder.

3.6 Cleaning electrodes

Before chip run and after completion of each chip run, the electrodes of the Agilent 2100 Bioanalyzer were cleaned to prevent contamination between runs. The electrode cleaner chip was filled with 350 µL of deionized water, inserted into the Bioanalyzer and the electrodes were soaked for 10 seconds to remove residual gel and sample components. Following cleaning, residual water was allowed to evaporate from the electrodes before the next chip run.

3.7. Statistical analysis

Statistical analysis and graphical visualisation of protein distribution data and rheological relationships were performed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) together with the Analysis ToolPak add-in. Correlation analysis and linear regression were used to evaluate the relationships between protein fractions and rheological parameters. t-tests were performed to compare the quantified levels of the five major milk proteins (α_s-CN, β-CN, κ-CN, β-LG, and α-LA) obtained using microfluidic chip electrophoresis and LC-HRMS Correlograms were additionally generated for visualisation of correlation patterns.

4. Results and Discussion

4.1. General electropherogram properties

Under reducing conditions, traditional SDS-PAGE separates skim milk proteins in order of increasing molecular mass: α-LA < β-LG < κ-CN < β-CN < α_{s1}-CN < α_{s2}-CN (Costa et al., 2014).

The electropherograms generated by the Agilent 2100 Bioanalyzer using the Protein 80 kit consistently showed five major protein fractions across all skim milk samples. Figure 4 shows a representative electropherogram of sample 1401-2.

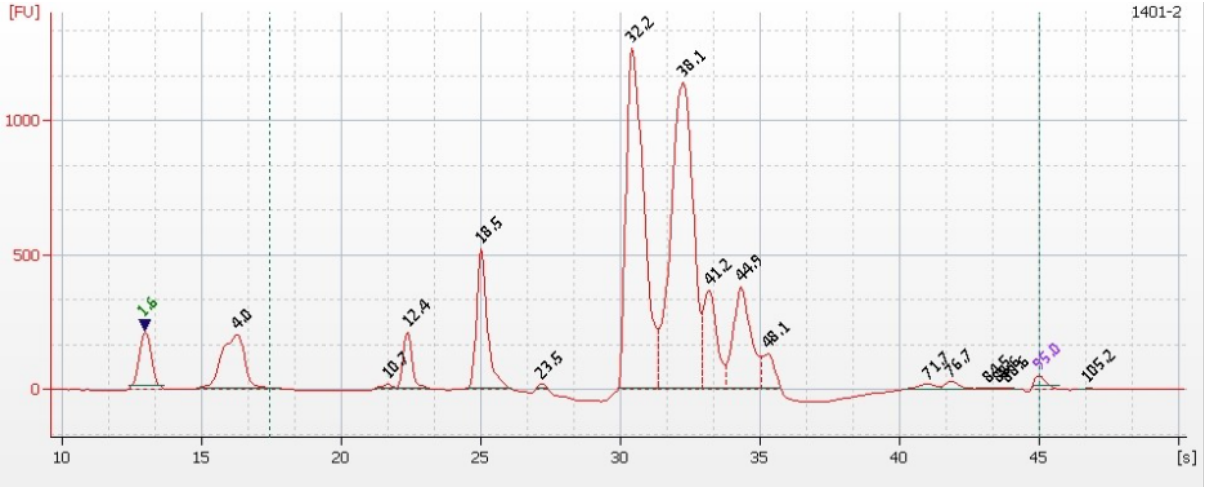


Fig. 4. *Representative electropherogram of skim milk sample 1401-2 analysed by the Agilent 2100 Bioanalyzer using the Protein 80 kit. Five major milk protein fractions are resolved: α-Lactalbumin (α-LA, ≈ 12.4 kDa), β-Lactoglobulin (β-LG, ≈ 18.5 kDa), β-CN(≈ 32.2 kDa), α_s-CN(≈ 38.1 kDa), and κ-CN(≈ 44.9 kDa). Lower (1.6 kDa) and upper (95 kDa) markers serve as internal migration time standards.*

The whey proteins were well resolved, with α-LA migrating first, followed by β-lactoglobulin. The casein

fractions were also separated, though with some peak overlap, and α_{s1}-CN and α_{s2}-CN could not be fully distinguished. Among the caseins, β-CN migrated first, followed by α_s-CN, then κ-CN. This peak overlap may interfere with quantification and affect the accuracy of protein concentration estimates, which is consistent with previous findings using the same method for bovine milk (Anema, 2009; Nitsche, 2016). Several minor peaks were also detected. Based on comparison with apparent molecular masses of individual milk protein standards reported by Anema (2009), these peaks were tentatively identified as γ-CN (approximately 10.7 kDa), IgG heavy chain (approximately 71.7 kDa), and BSA (approximately 76.7 kDa).

With the bioanalyzer runs, some of the peaks were difficult to separate clearly. This was mostly noticeable in the α-CN and κ-CN regions where peaks sometimes overlapped each other. Consequently, some inconsistencies during sample preparation could affect the final protein percentages. Even though the bioanalyzer method was less consistent, some of the same patterns could still be seen when compared with the LC-HRMS results. So, the method was still able to pick up differences between samples, even if the exact values were not always reproducible.

The assignment of peaks in milk protein electropherograms has not been entirely consistent across studies. Anema (2009) tentatively attributed the peak eluting immediately prior to α-LA (12 kDa) to γ-CN, based on comparison with conventional SDS-PAGE migration patterns. In contrast, Salunke et al. (2021) reported a distinct elution sequence by capillary gel electrophoresis, in which proteose peptones migrated ahead of α-LA, followed by β-LG, γ-CN, β-CN, α_s-CN, and κ-CN in order of decreasing mobility. This discrepancy may partly reflect the heterogeneity of the γ-CN family. The predominant fraction, γ1-CN (residues 29–209 of β-CN), has an estimated molecular weight of approximately 20 kDa (la Gatta et al., 2023), which would place it between β-lactoglobulin (~18 kDa) and β-CN (~32 kDa) under chip electrophoresis conditions, which consistent with the elution order described by Salunke et al. (2021). The smaller fractions, γ2-CN (~12 kDa) and γ3-CN (~11.5 kDa) (la Gatta et al., 2023), possess molecular weights below that of α-lactalbumin (~14 kDa), and may therefore migrate in the region tentatively assigned to γ-CN by Anema (2009).

While observing that a portion of β-CN was hydrolysed into γ-CN, a statistically significant difference in β-CN content was observed between the two analytical platforms ($p = 7.17 \times 10^{-10}$), with the Bioanalyzer yielding a lower mean value (34.22%) than LC-HRMS (38.59%) as seen in Table 4. While inter-method variability in protein quantification cannot be excluded as a contributing factor, one possible explanation relates to the time interval between the two analyses, LC-HRMS was performed in May 2025, whereas chip electrophoresis was conducted approximately one year later in 2026, during which the samples were stored without prior heat treatment to inactivate indigenous enzymes. β-CN primary proteolysis is predominantly mediated by plasmin, resulting in the formation of γ-CN fragments (la Gatta et al., 2023; Nielsen, 2002), and plasmin activity has been shown to persist under freezing conditions, with measurable increases in γ-CN fractions reported in samples stored at −20 °C over 28 days (la Gatta et al., 2023). It is therefore plausible that a proportion of β-CN was hydrolysed to γ-CN fragments during the storage interval, which would not be quantified as β-CN in the subsequent chip electrophoresis analysis. However, this hypothesis would require further verification, for example through the direct quantification of γ-CN fractions at both time

points.

Table 4. *Comparison of major casein and whey protein fractions between Agilent 2100 Bioanalyzer and LC-HRMS using paired t-test*

Protein	Bioanalyzer	LC-HRMS	P(T<=t) two-tail[a]
	Mean ± SD	Mean ± SD	
α-LA	3.58 ± 0.53	3.37 ± 0.27	0.11753
β-LG	9.84 ±1.35	8.72 ± 0.66	0.00192
β-CN	34.22 ± 1.91	38.59 ± 0.57	< 0.001
αs-CN	38.21 ± 2.13	37.92 ± 0.75	0.56082
κ-CN	9.44 ± 1.65	8.79 ± 0.24	0.092

[a] *When the p-value < 0.05, it means significant difference*

Table 5. *Expected molecular weight of the five analysed milk protein fractions compared to the apparent molecular weight as determined by the Agilent 2100 Bioanalyzer Protein 80 kit and SDS-PAGE.*

Milk protein	Expected MW (kDa)[a]	Bioanalyzer apparent MW (kDa)[b]	SDS-PAGE apparent MW (kDa)[d]
	Mean ± SD		Mean ± SD
α-LA	14	12.4 ± 0.00	13 ± 3
β-Lactoglobulin	18	18.5 ± 0.00	19 ± 2
β-CN	24	32.2 ± 0.06	27 ± 4
α_s-CN	23 / 25[c]	38.1 ± 0.06	30 ± 3 / 35 ± 6 [c]
κ-CN	19	44.9 ± 0.10	24 ± 4

[a.] *Expected values from Huppertz, T. et al. (2025). Dairy Science and Technology (3 ed.);*

[b.] *apparent values from Bioanalyzer (sample 1401-2);*

[c.] *αs1-CN/ α s2-CN;*

[d.] *From Anema, 2009*

The apparent molecular masses of αs-CN and β-CN estimated from the microfluidic chip analysis were higher than their known sequence-based values (Table 5). This observation aligns with what has been reported for these proteins in traditional SDS-PAGE (Anema, 2009; Nitsche, 2016).

Creamer and Richardson (1984) demonstrated that this overestimation is not caused by reduced SDS binding, but results from the unusually large hydrodynamic size of these proteins. The high density of negatively charged residues concentrated in certain segments of αs1-CN induces electrostatic repulsion within the polypeptide chain, resulting in an extended conformation and a larger effective size under electrophoretic conditions. Both phosphoserine residues and carboxyl groups of acidic residues contribute to this high local

charge density, although charge distribution along the chain is considered the primary factor.

The broad peak profile and apparent molecular weight overestimation of κ-CN observed in electrophoretic analyses can be largely attributed to its O-glycosylation. κ-CN exists as multiple glycoforms carrying varying numbers of O-glycan chains, which results in a heterogeneous population of molecules with different hydrodynamic sizes, thereby producing a broad rather than a discrete peak (Salunke et al., 2021). The additional mass contributed by these carbohydrate moieties further accounts for the upward shift in apparent molecular weight relative to the theoretical protein backbone. As shown in Table 5, the theoretical molecular weight of κ-CN is 19 kDa, whereas the apparent molecular weights determined by the Agilent 2100 Bioanalyzer and SDS-PAGE were 44.9 kDa and 24 kDa, respectively, reflecting overestimation on both platforms. Notably, non-glycosylated proteins such as α-lactalbumin and β-lactoglobulin showed apparent molecular weights consistent with their theoretical values on both platforms, further confirming that the observed shifts are glycosylation-dependent. However, glycosylation alone does not fully explain all discrepancies observed across analytical platforms. Specifically, while κ-CN (24 kDa) migrates ahead of β-CN (27 kDa) under SDS-PAGE conditions, the migration order is reversed on the Agilent 2100 Bioanalyzer, where κ-CN (44.9 kDa) elutes after both β-CN (32.2 kDa) and αs-CN (38.1 kDa). The mechanistic basis for this platform-dependent reversal in migration order, as well as the comparatively weak peak intensity of trace milk proteins observed on the microfluidic chip platform, remains unclear. Notably, analogous migration anomalies have been reported for other glycoproteins under CE-SDS versus SDS-PAGE conditions (Wang et al., 2020), suggesting that interactions between glycan chains and the separation matrix may introduce platform-specific effects. The three methods (Salunke et al., 2021; Anema, 2009; Agilent Technologies, 2011) were selected for comparison because they share the same fundamental separation principle, in which protein–SDS complexes are resolved according to molecular size through a sieving matrix (Wiesner et al., 2021).

Although the major protein fractions could be identified, complete peak separation was not always achieved. In particular, the α-CN region appeared as a broad distribution rather than a single sharp peak, and partial overlap between adjacent casein fractions was occasionally observed. This limitation may contribute to variability in protein quantification and represents an inherent challenge when analyzing heterogeneous casein populations using SDS-based microfluidic electrophoresis.

As shown in Table 5, α-lactalbumin (α-LA) and β-lactoglobulin (β-LG) showed apparent molecular weights of 12.4 kDa and 18.5 kDa on the Agilent 2100 Bioanalyzer, and 13 kDa and 19 kDa on SDS-PAGE, respectively, both closely aligned with their theoretical values of 14.2 kDa and 18.3 kDa. Neither protein exhibited substantial overestimation observed for the caseins. This can be attributed to the absence of post-translational modifications (PTMs) known to interfere with electrophoretic migration. As shown in Table 3, both α-LA and β-LG carry no phosphoserine residues and no hexose-linked glycans, which are the primary PTMs responsible for apparent molecular weight inflation in caseins. Without these modifications, SDS binds uniformly along the polypeptide backbone, and migration behaviour is governed predominantly by molecular size, resulting in close agreement between apparent and theoretical molecular weights across both platforms. The slight underestimation observed for α-LA may be attributed to reduced measurement

precision at the lower end of the molecular weight range, given its small size of 14.2 kDa (Anema, 2009). Although α-LA possesses the highest disulfide bond density among the milk proteins analyzed, with four S–S linkages per molecule (Table 3), the contribution of this structural feature to migration under reducing conditions is considered negligible compared to the calibration precision limitation at low molecular weights. In contrast, β-LG, with two disulfide bonds and one free thiol (Table 3), and similarly lacking glycosylation and phosphorylation, showed apparent molecular weights in close agreement with its theoretical value on both platforms, consistent with predictable size-based separation behaviour.

4.1.1. Quantification of Protein Fractions

Table 6. *Quantification of Protein Fractions from Agilent 2100 Bioanalyzer and LC-HRMS*

Protein	Bioanalyzer	LC-HRMS[a]
	Mean ± SD	Mean
α-LA	3.63 ± 0.06	3.74
β-LG	9.97 ± 0.06	7.27
β-CN	32.7 ± 0.26	38.38
α_s-CN	41.53 ± 0.21	38.39[b]
κ-C	8.83 ± 0.06	8.9
Whey	13.6	11.1
Casein	83.1	85.7
Total	96.7	96.7

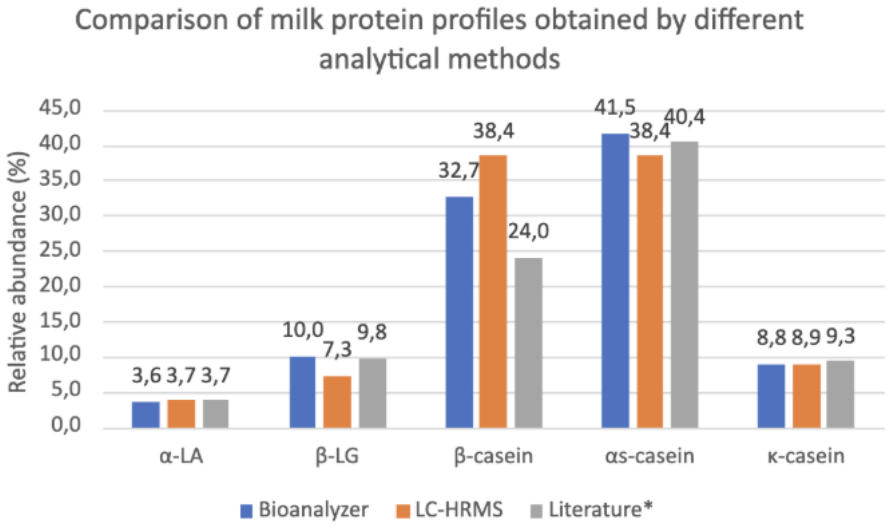
[a] *Data from Arla Innovation Centre*

[b] *Combine α_{s1}-CN and α_{s2}-CN together*

The protein distribution observed in sample 1401-2 is presented here as a representative illustration of the overall pattern. The casein fractions collectively accounted for 83.1% of the total quantified protein signal in sample 1401, while the whey proteins contributed 13.6%. The five proteins together represented 96.6% of all detected fluorescence signal, indicating that the Protein 80 assay captures the dominant protein components of bovine skim milk. The remaining approximately 3.4% most likely corresponds to minor proteins, including bovine serum albumin, lactoferrin, immunoglobulins, and proteose peptones, present at low concentrations and not resolved under the conditions applied. The overall distribution, with caseins as the clearly dominant fraction, is consistent with the established protein composition of bovine milk (Ribadeau-Dumas & Grappin, 1989; Nitsche, 2016).

In the electropherogram of sample 1401-2, α_s-CN appeared as two adjacent peaks at approximately 38.1 kDa and 41.2 kDa (Fig. 4). Based on the findings of Anema (2009) who used the same chip method, who reported that the peak was absent when α_{s1}-CN alone was used as a reference standard in skim milk analysis, this peak might be attributed to α_{s2}-CN. As the two fractions co-migrate in proximity under the chip-based separation conditions and cannot be fully resolved, their peak areas were combined and reported as total α_s-CN in Table 4.

Figure 5. *Comparison of milk protein relative abundance (% of total quantified protein) of sample 1401 obtained by the Agilent 2100 Bioanalyzer, LC-HRMS, and literature reference values.*



* Literature values are derived from Huppertz, T., Dairy Science and Technology, 3rd ed., CRC Press, 2025. LC-HRMS data provided by Arla Innovation Center.

The relative protein distribution obtained by the Agilent 2100 Bioanalyzer was compared against LC-HRMS and literature reference values, as shown in Figure 5. For α-LA, αs-casein, and κ-casein, the Bioanalyzer results were in good agreement with both LC-HRMS and literature values, and no statistically significant differences were detected between the two methods ($p = 0.118$, 0.561, and 0.092, respectively; Table 4). The β-casein fraction, however, showed lower relative abundance in both the Bioanalyzer and LC-HRMS measurements compared to the literature reference. As both methods were applied to the same sample, this discrepancy is unlikely to reflect a methodological limitation and more plausibly reflects sample-specific variation. Furthermore, the statistically significant difference in β-casein between the Bioanalyzer and LC-HRMS ($p = 7.17E-10$, Table 4), where the Bioanalyzer yielded a notably lower value (34.22 ± 1.91%) compared to LC-HRMS (38.59 ± 0.57%), is attributed to the fact that the Bioanalyzer measurements were conducted approximately one year after the LC-HRMS analysis. This time gap likely resulted in proteolytic degradation of β-casein within the stored sample, rather than representing a difference in analytical performance between the two methods. For β-LG, the Bioanalyzer yielded a slightly higher relative abundance than LC-HRMS, and this difference reached statistical significance ($p = 0.00192$, Table 4); however, both values remained within a comparable range and the reason for this discrepancy is not fully understood.

4.1.2. Reproducibility

Within-run reproducibility was assessed using three replicate injections of sample 1401 on the same chip (Table 7). The coefficient of variation (CV) across all five protein fractions ranged from 5.1% (α-LA) to 6.2% (β-CN), the reproducibility within a single chip was deemed very good with coefficients of variation (CVs) less than 10% for all milk proteins. However, it should be noted that only within-run reproducibility was

evaluated in this study. Between-chip reproducibility, which accounts for variability introduced by chip-to-chip differences in electrode performance and sample loading, was not assessed. Future studies should include between-chip replicates to provide a more comprehensive evaluation of the method's reproducibility.

Table 7. *Within-run reproducibility of the Agilent 2100 Bioanalyzer for five skim milk protein fractions in sample Farm 1401 (n = 3 replicate injections on the same chip). CV = coefficient of variation.*

Protein	**Calculated concentration**	**Reproducibility within chip**	
	$mgmL^{-1}$	Peak area	CV (%)
α-LA	1.3	74.5 ± 3.8	5.1
β-LG	3.5	227.1 ± 14.1	6.2
β-CN	11.5	899.5 ± 51.3	5.7
α_s-CN	14.6	1017.1 ± 54.3	5.3
κ-CN	3.1	272.9 ± 16.2	5.9

4.2. Whey Protein Analysis

At 1:5 dilution, WPM and WUPM each yielded two detected peaks corresponding to α-La and β-Lg, at apparent molecular weights consistent with the expected values for these proteins on the Protein 80 platform (Table 8; Nitsche, 2016). No additional peaks were detected in either sample at this dilution. Both WPM and WUPM showed peaks of α-lactalbumin at 12.6 to 12.7 kDa and β-lactoglobulin at 18.3 to 18.7 kDa. The whey abundance was also similar between WPM and WUPM, with α-lactalbumin representing 29% and 29.8%, while β-lactoglobulin represented 64% and 67%, respectively. 1:5 dilution showed that it can reduce overlapping regions of proteins.

Table 8. *Detected whey protein masses and relative abundances in 1:5 diluted whey samples.*
(Abbreviations: WPM, whey from pasteurised skimmed milk; WUPM, whey from unpasteurised skimmed milk.)

WPM		**WUPM**		**Known protein type**
Size (kDa)	**%**	**Size (kDa)**	**%**	
12.6 ± 0.2	29.0 ± 0.8	12.7 ± 0.1	29.8 ± 0.0	α-LA
18.3 ± 0.2	64.0 ± 0.8	18.7 ± 0.1	67.0 ± 1.1	β-LG

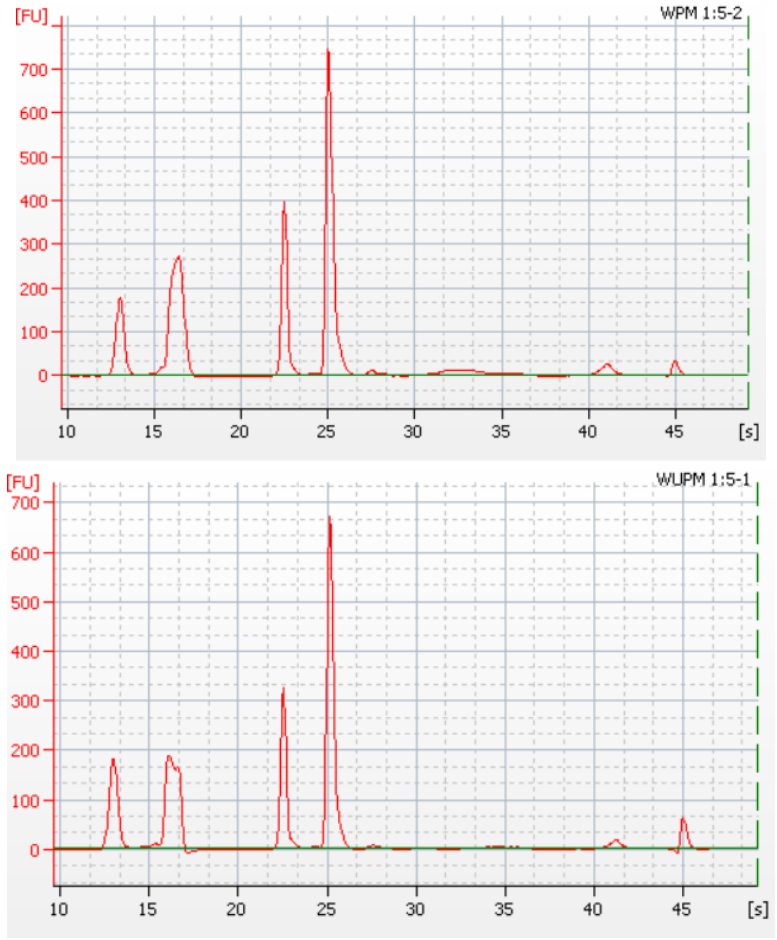


Figure 6. *Representative electropherograms obtained by microfluidic chip electrophoresis of whey from pasteurised skim milk (WPM) and unpasteurised skim milk (WUPM) at 1:5 dilution.*

At 1:2 dilution, an additional peak around 16.3 to 16.4 kDa was also detected, although their abundances are significantly different with a lower percentage or nearly absent in WUPM (Table 9). The whey of pasteurised skimmed milk (WPM) showed 22.1% of α-LA and 51.6% of β-LG whereas WUPM has a higher percentage of both α-LA (33.5%) and β-LG (60.5%). The relative abundances of α-La and β-Lg also differed from the 1:5 values in both sample types.

Table 9. *Detected whey protein masses and relative abundance in 1:2 diluted skimmed milk samples. (Abbreviations: WPM, whey from pasteurised skimmed milk; WUPM, whey from unpasteurised skimmed milk.)*

WPM		**WUPM**		**Known protein type**
Size (kDa)	%	Size (kDa)	%	
12.5 ± 0.2	22.1 ± 1.4	2.5 ± 0.2	33.5 ± 0.4	α-LA
16.3 ± 0.3	9.8 ± 0.3	6.4 ± 0.2	0.6 ± 0.3	
18.4 ± 0.2	51.6 ± 3.2	8.3 ± 0.1	60.5 ± 1.4	β-LG

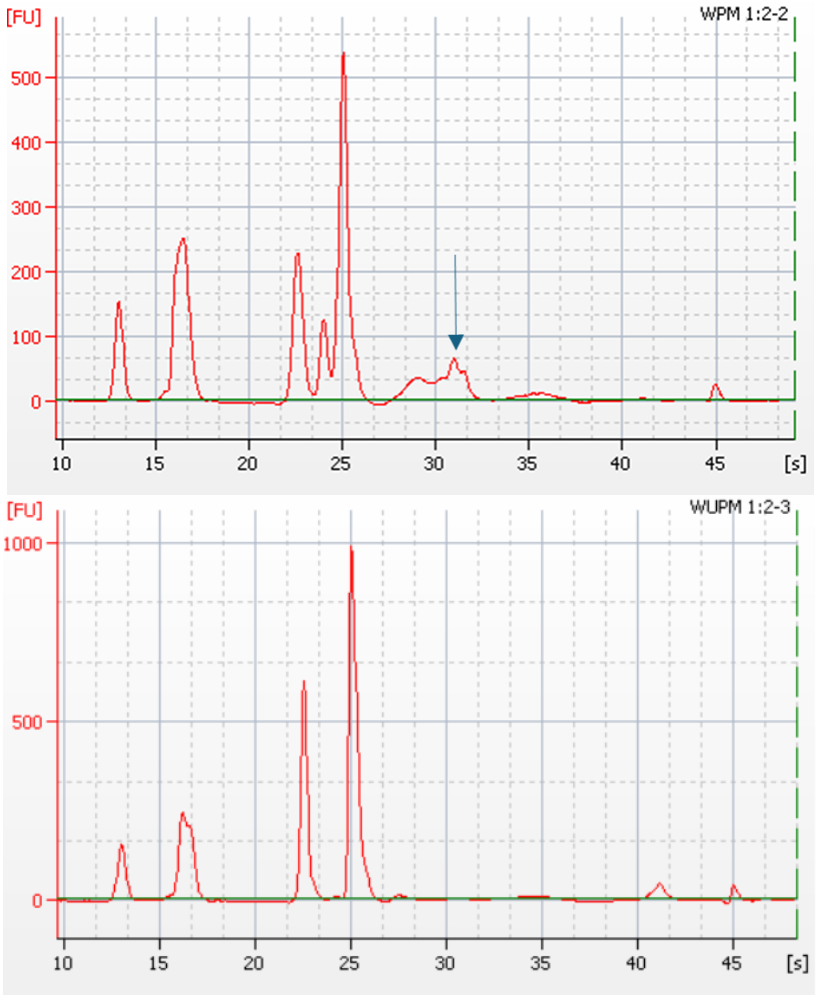


Figure 7. *Representative electropherograms of whey protein fractions from pasteurised skim milk (WPM) and unpasteurised skim milk (WUPM) at 1:2 dilution obtained by microfluidic chip electrophoresis.*

4.3. Dilution selection

When whey samples were analysed without dilution, protein peaks exceeded the linear detection range of the Agilent 2100 Bioanalyzer, producing measurement errors in all three sample types. Two dilution factors — 1:2 and 1:5 (v/v) in deionised water — were therefore chosen and evaluated. At both dilutions, α-lactalbumin (α-La) and β-lactoglobulin (β-Lg) were detected as distinct peaks within their expected apparent molecular weight ranges on the Protein 80 platform (as described in Section 4.1). In 1:2 dilution, strong peak signals were observed particularly in the whey proteins and a small peak in κ-CN regions. An additional peak around was also detected, this might suggest instability of peak or overlapping of protein fractions. However, the electropherograms were less stable than suggested residual milk components such as fat, milk serum and overlapping casein fractions. Residual milk components, including fat and milk serum constituents, may have contributed to peak instability and overlapping protein fractions. However, the present study did not specifically evaluate the extent of such effects. The 1:5 diluted samples in table 1 showed clear, distinguishable peaks with no unknown middle peaks. They showed that 1:5 can reduce overlapping regions of proteins. In conclusion, the 1:2 dilution had better sensitivity for κ-

CN while the 1:5 dilution provided a cleaner separation and more reliable whey protein profiling. A final dilution of 1:5 (v/v) was therefore applied to all whey samples for subsequent analysis.

4.3.1. WPM and WUPM: Major whey proteins and the unidentified 16.3 kDa peak

At 1:5 dilution, the detection of only α-La and β-Lg in both WPM and WUPM, at apparent molecular weights consistent with the Protein 80 platform reference values (Nitsche, 2016), confirms that the method can reliably identify the two dominant whey proteins under these conditions. The similar relative abundances of α-La and β-Lg between WPM and WUPM suggest broadly comparable whey protein profiles. However, it should be noted that WPM (from commercially low-pasteurised Arla Ko® milk) and WUPM (from dairy silo milk) originate from different milk sources, which differ in breed, herd, feed, and processing history in addition to pasteurisation status. Direct attribution of any differences between WPM and WUPM solely to pasteurisation is therefore not possible from the present data.

The additional peak at 16.3 kDa detected in WPM at 1:2 dilution (9.8 ± 0.3%) but not in WUPM (0.6 ± 0.3%) is of uncertain identity. Its molecular size does not correspond to the expected apparent molecular weights of the major milk proteins on the Protein 80 platform (Section 4.1; Nitsche, 2016). Importantly, low-temperature short-time pasteurisation is not expected to cause significant structural modification of α-La or β-Lg. Shuang et al. (2025) reported that neither protein undergoes appreciable lactosylation or denaturation below 85°C under HTST conditions, and their band intensities on SDS-PAGE were maintained after standard pasteurisation. The 16.3 kDa peak is therefore unlikely to represent a pasteurisation-derived denaturation product of the major whey proteins. A peak of similar size (~16.5 kDa) has been previously observed in rennet-processed whey analysed on the same platform and was tentatively attributed to proteolytic fragments generated by rennet activity (Blixth, 2022). The disappearance of this peak at 1:5 dilution suggests it represents a low-abundance component falling below the detection threshold at greater dilution.

Although the theoretical molecular weight (MW) of glycomacropeptide (GMP) is approximately 7 kDa, its apparent molecular weight observed during gel filtration changes depending on pH conditions. At pH 7, the apparent MW ranges from about 20 to 50 kDa, whereas at pH 3.5 it decreases to approximately 10–30 kDa. This difference indicates that GMP does not behave as a compact 7 kDa molecule in solution (Figure 7). Instead, factors such as molecular aggregation, conformational changes, charge effects, and extensive glycosylation increase its hydrodynamic volume, causing it to appear larger (Kawasaki, et al., 1993).

4.3.2. Limitations

Several limitations of the present study should be acknowledged. First, as discussed in Section 4.1, the Agilent 2100 Bioanalyzer tends to overestimate the relative abundance of β-Lg, which affects the quantitative interpretation of the whey protein data presented here. Second, LC-HRMS data were not available for all samples, limiting direct validation of the relative abundance values obtained by microfluidic chip electrophoresis. Third, WPM and WUPM originate from different milk sources, limiting the interpretation of differences between these two sample types. Fourth, the identity of the

16.3 kDa peak observed in WPM (at 1:2 dilution) and WWM (at 1:5 dilution) remains unresolved and requires further investigation.

4.4. Correlation properties between different proteins proportions and rheological properties

Seventeen farms and four silos skimmed milk samples were analysed. Data from farm 6 was not available, and data from farm 297 is an outlier case as it had a higher gel strength than the others. Therefore, the data below were analysed with and without farm 297.

4.4.1. Casein data from microfluidic chip correlation with rheological properties

4.4.1.1. κ-CN

κ-CN showed a weak positive relationship with coagulation time with R^2 of 0.035 and increased slightly after inclusion of sample 297. This suggests that κ-CN content alone has limited influence on coagulation time. The weak correlation indicates that coagulation time is influenced by different factors beyond κ-CN such as pH concentrations, calcium balance and casein micelle organization (Karlsson, et al., 2005).

Among all casein fractions, κ-CN showed the strongest relationship with yield stress. Inclusion of sample 297 increased the R^2 value from 0.160 to 0.270. Sample 297 was characterized by both a relatively high κ-CN content and an unusually high yield stress, which contributed substantially to the observed increase in correlation strength. Nevertheless, even after inclusion of the outlier, the R^2 value remained relatively low, indicating that κ-CN alone had a limited effect on yield stress. The results therefore suggest a possible contribution of κ-CN to gel network strength and structural resistance during coagulation, but additional factors are likely involved.

A weak positive relationship was also observed between κ-CN content and storage modulus at 2.5 times the gelation time ($G'_{2.5}tg$). Inclusion of sample 297 increased the R^2 value from 0.132 to 0.251. Like the yield stress results, the increase demonstrates the influence of the outlier on the correlation. Although κ-CN showed the highest correlation among the casein fractions, the relatively low R^2 values suggest that κ-CN is only one of several factors contributing to gel development and elastic network formation during coagulation.

Lastly, a weak positive correlation relationship was observed between κ-CN content and storage modulus after 30 minutes of coagulation (G'_{30}). Without sample 297, the relationship was weak ($R^2 = 0.079$). However, after the inclusion of sample 297, the R^2 value increased to 0.208, demonstrating the sensitivity of the correlation to the outlier. Therefore, the observed trend should be interpreted with caution. Higher κ-CN content may be associated with increased gel firmness after 30 minutes of coagulation, the low explanatory power of the model indicates that other compositional and processing factors also contribute substantially to the variation in G'_{30}.

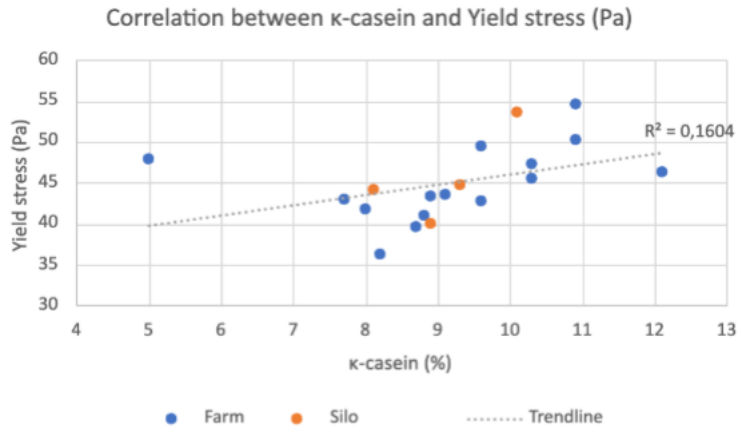


Figure 8. *Correlation plot between κ-CN percentage in microfluidic chip data and yield stress (Pa) from rheometer data.*

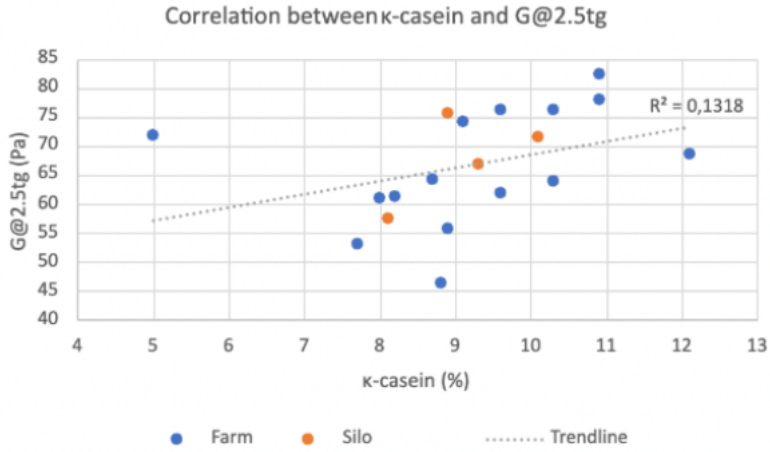


Figure 9. *Correlation plot between κ-CN percentage in microfluidic chip data and G'$_{2.5}$tg (Pa) from rheometer data.*

4.4.1.2. α-CN

α-CN correlation plot in Figure 10 showed the strongest positive relationship with coagulation time in the casein dataset. A strong positive correlation was observed in both correlations and sample 297 had minimal effects. α-CN is a major component of the casein micelle, over 50%, and contributes to micellar organization, hydration, and protein-protein interactions during coagulation (O'Mahony and Fox, 2013). Therefore, variations in α-CN content may influence the rate of micelle destabilization and gel network formation.

The R^2 values indicated little association between α-CN content and yield stress, G'$_{2.5}$tg, or G'$_{30}$. This indicates α-CN had less contribution to gel firmness at equivalent coagulation stages and after thirty minutes.

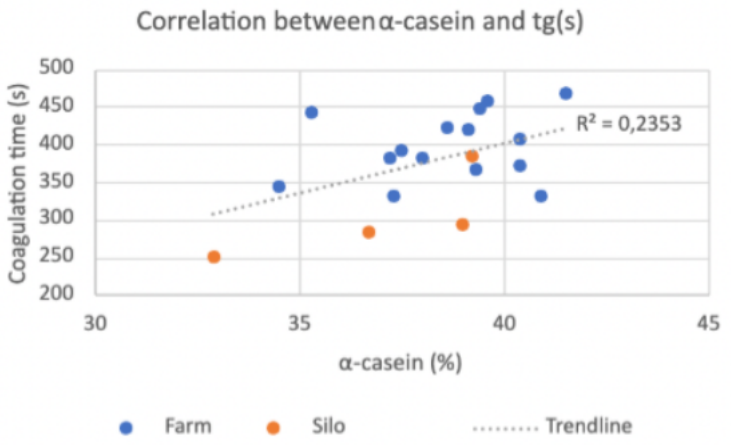


Figure 10. *Correlation plot between α-CN percentage in microfluidic chip data and coagulation time (s) from rheometer data.*

4.4.1.3. β-CN

β-CN overall had a weak negative relationship with coagulation time, no significant relationship was observed with yield stress and a weak positive relationship with $G'_{2.5}tg$. Higher β-CN levels were associated with slightly shorter coagulation times and inclusion of sample 297 reduced the correlation strength. κ-CN showed weak to moderate positive correlations with $G'_{2.5}tg$ and yield stress, suggesting a potential contribution to gel firmness development. However, β-CN demonstrated a stronger positive relationship with G'_{30}, indicating that β-CN may contribute more than κ-CN to the elastic gel network formation at 30 minutes.

4.4.2. Comparison of correlation patterns between microfluidic chip electrophoresis and LC-HRMS

The correlograms presented in Figure 11 were generated after exclusion of sample 297 due to its strong influence on several regression models and overall correlation patterns. The correlation patterns obtained from the two analytical methods were not completely identical, although several similar trends could still be observed. In the bioanalyzer dataset, α-CN showed the clearest positive relationship with gelation time, while β-CN appeared to be more closely related to G'_{30}. κ-CN, on the other hand, showed more noticeable positive relationships with $G'_{2.5}tg$ and yield stress.

The LC-HRMS correlations showed a somewhat different pattern. Here, κ-CN demonstrated the strongest positive relationship with gelation time, whereas β-CN again showed the most visible positive trends with the gel firmness parameters. κ-CN seemed to show stronger associations with coagulation initiation, especially in the LC-HRMS dataset. This agrees with the known role of κ-CN in stabilizing the surface of casein micelles and in the early stages of rennet coagulation following enzymatic cleavage of the micelle surface layer (Dalgleish, 1992). Even though many of the correlations remained relatively weak, the results from both methods suggest that the different casein fractions may contribute differently during coagulation and gel development. The differences observed between the two analytical methods may partly reflect the differences in protein profiles obtained by each technique. In the present study, microfluidic chip electrophoresis showed lower resolution in some protein regions, particularly for α-CN and κ-CN, where

peak overlap occasionally occurred. In contrast, LC-HRMS provided more detailed protein separation and quantification. These differences may explain why α-CN showed the strongest association with gelation time in the bioanalyzer dataset, whereas κ-CN displayed stronger relationships with gelation time in the LC-HRMS dataset. Therefore, variations in protein quantification between the two methods likely contributed to the observed differences in correlation patterns.

Overall, neither method produced consistently strong correlations for all rheological parameters. This was not unexpected, since milk coagulation is affected by multiple compositional and environmental factors in addition to protein composition alone. However, both analytical approaches were able to detect biologically meaningful trends between casein fractions and coagulation behaviour, suggesting that they may have potential tools for studying milk coagulation properties.

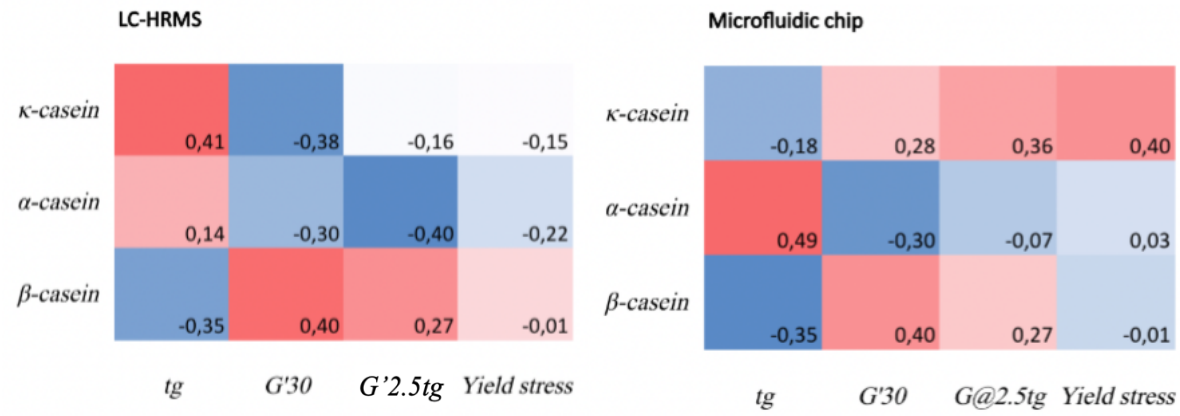


Figure 11. *Correlograms comparing LC-HRMS and microfluidic chips with Bioanalyzer.*

5. Conclusion

This study evaluated microfluidic chip electrophoresis using the Agilent 2100 Bioanalyzer and Protein 80 kit as a rapid method for milk protein profiling and compared the results with LC-HRMS. The method successfully detected the major milk protein fractions, including αs-casein, β-casein, κ-casein, β-lactoglobulin, and α-lactalbumin, and demonstrated good within-run reproducibility across repeated analyses. However, several limitations were identified, including overlapping protein peaks, differences in apparent molecular weight compared with theoretical values, and reduced ability to resolve protein isoforms and post-translational modifications relative to LC-HRMS.

Correlation analysis indicated that certain protein fractions, particularly κ-casein, showed associations with rheological properties related to rennet coagulation. However, these relationships were generally weak, suggesting that protein composition alone has a limited effect on coagulation behaviour and that additional factors, such as micelle structure, and processing conditions are also important.

Overall, the results demonstrate that microfluidic chip electrophoresis has potential as a rapid, automated, and low-sample-volume method for comparative milk protein profiling. Nevertheless, further optimization

and validation are required before the method can be considered a reliable quantitative alternative to LC-HRMS. In particular, the validation performed in this study was limited to within-chip reproducibility; between-chip reproducibility, between-day reproducibility, and between-operator variability remain to be evaluated in future studies. At its current stage, the Agilent 2100 Bioanalyzer is most suitable as a high-throughput screening tool for routine dairy applications rather than a replacement for high-resolution mass spectrometry.

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A. Appendix

A1. Gel-Like Image

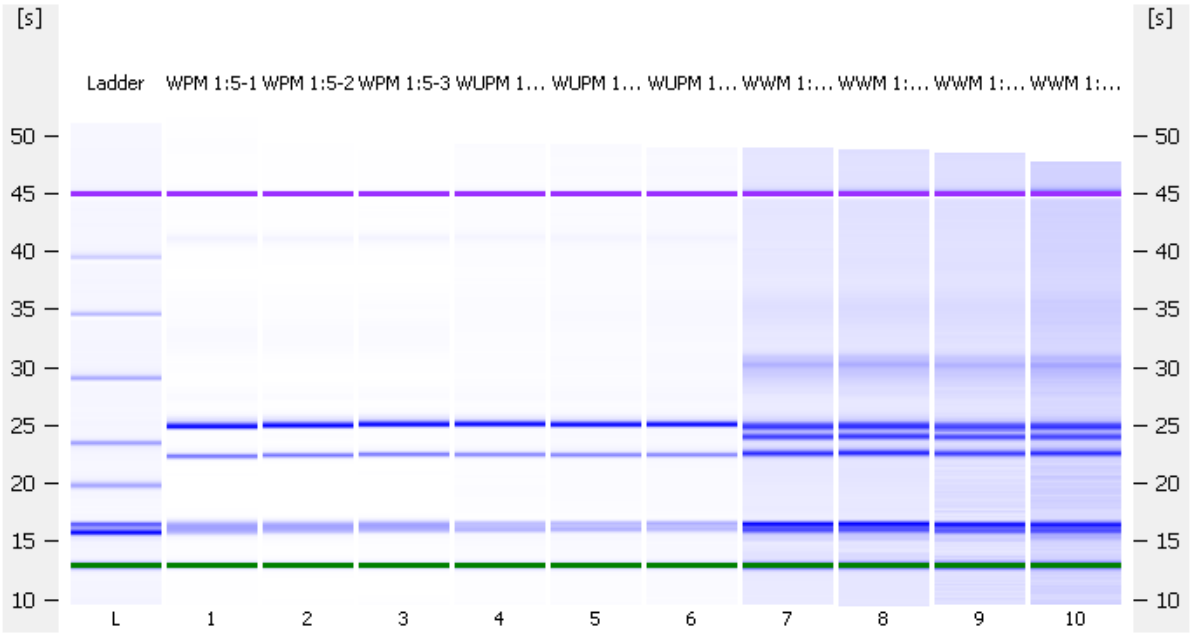

Figure A1. Example of a gel-like image taken from whey samples.

A2. Electropherogram ladder

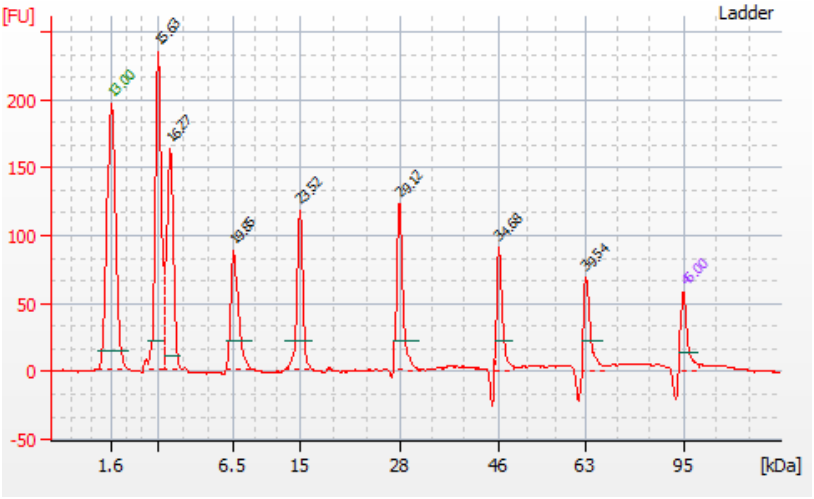

Figure A2. Example of a microfluidic chip ladder.

A3. Raw data of Farm 1401

Peak Size [kDa]	Rel. Conc. [ng/µl]	% Total	Observations	Area	Aligned Migration Time [s]
1.6	0	0	Lower Marker	109.1	13
4	0	0	System Peak	195.9	16.27
10.7	41	0.4		7.7	21.68
12.4	408.5	3.6	α-LA	78.6	22.38
18.5	1 124,00	10	β-LG	240.2	25.01
23.5	20	0.2		4.6	27.19
32.2	3664.7	32.6	β-CN	944.3	30.41
38.1	3943.5	35.1	αs-CN	1 075,00	32.25
41.2	732.1	6.5	αs-CN	205.2	33.19
44.9	997.3	8.9	κ-CN	288.8	34.33
48.1	215.3	1.9		64	35.28
71.7	40.4	0.4		13.9	41.03
76.7	48.2	0.4		16.9	41.87
84.5	7.7	0.1		2.8	43.21
86.6	2	0		0.7	43.56
88.6	3.7	-0.1		1.3	43.91
95	60	0	Upper Marker	22.6	45
105.2	0	0		1.2	46.74

Figure A3. Raw data table for sample Farm 1401.

A.4. Correlation summary table

Table A4. Summary table to compare casein fractions with different rheological properties.

Casein	**Rheological property**	**Trend without 297**	**R^2 without 297**	**Trend with 297**	**R^2 with 297**
κ-casein	tg	Weak +	0.035	Weak +	0.043
κ-casein	G'_{30}	Weak +	0.079	Moderate +	0.208
κ-casein	$G'_{2.5}$tg	Weak +	0.132	Moderate +	0.251
κ-casein	Yield stress	Weak +	0.160	Moderate +	0.270
α-casein	tg	Moderate +	0.235	Moderate +	0.234
α-casein	G'_{30}	Weak –	0.089	Very weak +	0.063
α-casein	$G'_{2.5}$tg	No relationship	0.005	No relationship	0.015
α-casein	Yield stress	No relationship	0.001	No relationship	0.004
β-casein	tg	Weak –	0.121	Weak –	0.073
β-casein	G'_{30}	Weak +	0.158	No relationship	0.015
β-casein	$G'_{2.5}$tg	Weak +	0.071	No relationship	0.002
β-casein	Yield stress	No relationship	0.00009	Very weak –	0.013

A.5. Average results of 21 samples by Agilent 2100 Bioanalyzer and LC-HRMS

Table A5. Relative abundance (%) of milk protein fractions in samples from individual farm and silo sources as determined by the Agilent 2100 Bioanalyzer and LC-HRMS.

	Whey						Casein							
	α-LA (%)		β-LG (%)		Total whey (%)		β-casein (%)		α-casein (%)		κ-casein (%)		Total casein (%)	
Milk source	Bioanalyzer	LC-HRMS	Bioanalyzer	LC-HRMS	Bioanalyzer	LC-HRMS	Bioanalyzer	LC-HRMS	Bioanalyzer	LC-HRMS	Bioanalyzer	LC-HRMS	Bioanalyzer	LC-HRMS
Farm_6	3,7±0,15	3,2	10,8±0,12	9,1	14,5	12,3	34,5±1,2	38,5	38,4±0,32	37,4	11,8±0,96	9	84,7	84,9
Farm_885	3,7±0,1	3,3	10,3±0,21	8,5	14,0	11,8	35,5±0,61	38,3	38,6±0,31	38,2	7,7±0,26	9	81,8	85,5
Farm_849	3±0	3,1	11,4±0,12	9,0	14,4	12,1	34,8±1,19	38,3	39,6±0,25	37,4	8,2±0,75	9,2	82,6	84,9
Farm_326	3,7±0,21	3,4	7,5±0,15	9,4	11,2	12,8	39,6±0,38	40	39,3±0,15	36,6	5±0,21	8,6	83,9	85,2
Farm_429	4,2±0,36	3,4	10,6±0,3	8,4	14,8	11,8	32,9±0,78	38,8	35,3±1,7	37,5	9,1±0,96	8,8	77,3	85,1
Farm_443	3,4±0,36	3,4	11,9±0,12	9,1	15,3	12,5	33,4±1,13	38	38±1,21	38,5	8,7±0,17	8,4	80,1	84,9
Farm_693	2,3±0,25	3,2	8,4±0,36	9,0	10,7	12,2	36,1±0,52	38,3	40,9±0,99	38,4	10,9±0,21	8,6	87,9	85,3
Farm_297	3,5±0,3	2,8	10,8±0	10,2	14,3	13,0	33,1±0,79	38	37,3±0,56	37	11,87±0,38	9,1	82,27	84,1
Farm_396	3,8±0,25	3,6	9,6±0,4	9,1	13,4	12,7	32,5±0,7	37,6	40,4±0,38	38	10,3±0,21	9	83,2	84,6
Farm_295	4,4±0,36	3,4	10,7±0,51	8,5	15,1	11,9	31,9±0,96	38,2	37,2±1,8	38,5	8,9±0,61	8,9	78	85,6
Farm_092	4,1±0,3	3,5	10,8±0,12	9,4	14,9	12,9	32,2±1,56	39,2	37,5±0,75	37,3	12,1±0,95	8,3	81,8	84,8
Farm_725	2,8±0,12	3,4	7±0,15	8,4	9,8	11,8	34,7±0,65	39,4	39,1±0,38	36,9	10,3±0,12	9,3	84,1	85,6
Farm_216	3,4±0,06	3	9,4±0,17	8,2	12,8	11,2	33,5±0,25	39,1	34,5±0,5	37,9	9,6±0,15	8,5	77,6	85,5
Farm_191	3,6±0,15	3,8	9,8±0,1	7,5	13,4	11,3	33,9±0,75	38,3	39,4±0,3	40	8±0,12	8,7	81,3	87
Farm_1401	3,6±0,06	3,7	10±0,06	7,3	13,6	11,0	32,7±0,26	38,4	41,5±0,21	38,4	8,8±0,06	8,9	83	85,7
Farm_181	4,3±0,2	3,1	10±0,31	8,4	14,3	11,5	31,7±1,02	39,1	40,4±0,51	38,1	10,9±2,49	8,9	83	86,1
Farm_116	3,2±0,06	3,9	8,5±0,51	8,2	11,7	12,1	36±1,33	38,6	37,3±2,06	38	9,6±1,62	8,8	82,9	85,4
Silo_GAS1(sil	4,1±0,25	3,2	10,7±0,32	8,9	14,8	12,1	33,5±0,61	39,1	36,7±1,17	37,7	10,1±0,75	8,7	80,3	85,5
Silo_FS1	2,9±0,23	3,3	9,7±0,36	9,2	12,6	12,5	35,6±0,81	38,3	32,9±0,15	37,8	9,3±0,06	8,7	77,8	84,8
Silo_FS2	3,4±0,38	3,6	7,4±0,26	8,5	10,8	12,1	36,9±0,61	38,8	39±1,24	38	8,9±0,46	8,7	84,8	85,5
Silo_SRBSIM	4±0,1	3,4	11±0	8,5	15,0	11,9	33,6±1,1	38,1	39,2±0,4	38,8	8,1±0,4	8,8	80,9	85,7