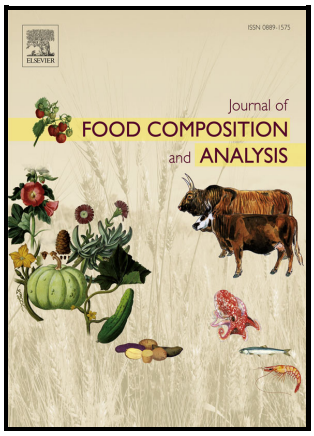

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Advancement of Milk Protein Analysis: From determination of total proteins to their identification and quantification by proteomic approaches

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ABSTRACT

Milk proteins are important components that confer several nutritional and functional properties to milk and dairy products. As these proteins have complex nature and wide variability, their analysis is not trivial and requires several methods to be used individually or

in combination. In this article, we provide a comprehensive review of conventional and more advanced methods used in the analysis of milk protein content, composition, and structural properties. Different traditional methods (mainly titration and spectrophotometric) are still used for the determination of total protein content, while chromatographic and electrophoretic techniques, enzyme-linked immunosorbent assays, and infra-red spectroscopy are used to separate, identify, and quantify individual proteins. For advanced structural identification of separated proteins, nuclear magnetic resonance, X-ray diffraction, and mass spectroscopic techniques can be used. Of these methods, the analyst may select those relevant to different applications in milk and dairy products research.

Keywords: Milk, proteins, milk products, analytical techniques, separation, quantification, structural identification

Introduction

Milk is a complex, heterogeneous emulsion of proteins, lactose, fat globules, minerals, and other minor constituents (Thompson, Boland & Singh, 2009). The diversity of milk proteins enables its transformation to a variety of products that provide a wide range of opportunities to the dairy industry (Starovojtova et al., 2020). Milk composition is determined by several factors including genetics, animal age, lactation stage, parity, health status, feed, and environmental conditions (Kalač & Samková, 2010). Milk contains an uncountable number of distinct proteins, which are categorized into caseins, (αs1, αs2, β, and κ-casein) and whey proteins (e.g. α-lactalbumin, β-lactoglobulin, bovine serum albumin, lactoferrin, immunoglobulin), as well as non-protein nitrogen components **(**Dupont, Croguennec & Pochet, 2018**)**. The most prominent milk proteins are illustrated in **Figure (1)**.

These and other micro-protein constituents of milk, including e.g. metal-binding proteins and enzymes, contribute to its technological and nutritional values (Lönnerdal, 2013).

, are especially interesting since they have been associated with several nutritional benefits as well as some limitations such as allergies (Haug, Høstmark, & Harstad, 2007). Besides delivering nitrogen and essential amino acids to humans and mammals (Duponta & Tome, 2020), several other health benefits are associated with milk protein consumption, including metabolic, anti-carcinogenic, antihypertensive, immune modulatory, and other effects (Davoodi et al., 2016). Further, milk proteins may facilitate the uptake of several essential nutrients such as trace elements and vitamins (Nagpal et al., 2011) and are sources of biologically active peptides (Tidona et al., 2009). However, β-lactoglobulin protein in milk trigger allergies in humans, notably in infants and children where bovine milk proteins constitute the most common food allergy (Warren et al., 2013).

The structure and properties of milk and milk products are determined by their protein content and composition (Runthala et al., 2023). Therefore, identifying and quantifying milk proteins is necessary for quality design and control, food labelling, and research and development. Consequently, milk protein analysis has become a subject of enormous interest to food technologists and nutritionists. Depending on the purpose, one can select from a wide range of analytical methods of milk proteins including spectroscopic, chromatographic, electrophoretic, and immune-histochemical. This article aims to provide a comprehensive review of various techniques currently being employed for the analysis of milk proteins, including traditional methods as well as advanced proteomic techniques. The principles of the different methods as well as their specialty and limitations are discussed. Furthermore, some important applications of the reviewed methods of protein analysis in milk and dairy product analyses are discussed.

2. Quantification of Total Protein Content by Titration and Spectroscopic Methods

2.1. Titration Methods

The determination of total proteins, *via* the analysis of total nitrogen content, is achieved using the reference method described by Kjeldahl (1883). This method is achieved by mixing the sample with K_2SO_4/H_2SO_4 and converting it to ammonium sulfate followed by the transformation of ammonium ions into ammonia gas by adding NaOH and boric acid. Finally, the ammonium gas is titrated with hydrochloric acid to provide the total nitrogen content, which is converted to crude protein content by multiplication by a conversion factor (typically 6.38 for milk) based on 15.67% nitrogen content in caseins. Because of this approximation, the Kjeldahl method may overestimate the protein contents in samples containing large amounts of non-protein nitrogen, typically 3-8% in whole milk and 25-30% in whey (Chiacchierini et al., 2003). The Kjeldahl method has high precision despite the bias that can be caused by using the conversion factor. In addition, this method is time-consuming and uses large amounts of aggressive and polluting chemicals. Therefore, other methods are preferred for determining the total milk proteins including the formol titration, spectroscopic methods, coulometry, and dye-binding procedures.

The formol titration involves reacting the $-NH_2$ groups of proteins, peptides, and amino acids with formaldehyde (HCHO) to create methylene amino acid derivatives ($-N=CH_2$), and titrating -COOH groups with 0.1N NaOH. No significant differences were found between the formol and Kjeldhal titration methods in the determination of goat milk proteins (Gomma, 2014). However, the formol titration method is easily implemented (James, 1995) and may be more accurate than the Kjeldahl method in samples containing non-protein nitrogen.

2.2. Spectrophotometric Methods

Compared with the Kjeldahl method, several spectrophotometric methods are used for fast, and sometimes more accurate, protein quantification in milk and dairy products (**Table 1**). Generally bovine serum albumin is used as a reference but these methods are sometimes calibrated for each protein composition to allow certain specificity and accuracy. Direct spectroscopic analysis of proteins is based on their UV absorption due to the presence of peptide bonds (at 205nm), to the aromatic amino acids (tyrosine (Tyr), tryptophan (Trp), and to small extent phenylalanine (Phe)), and the disulfide bonds of cysteine in their structure. The absorption spectra of the aromatic amino acids differ slightly, *e.g.* (i) Trp absorption peak is observed around 290 nm with fine structures up to 305 nm, (ii) Tyr absorption maximum occurs between 275 and 282 nm with a shoulder at longer wavelengths that is slightly obscured by Trp, and (iii) Phe has numerous less intense but sharper bands between 255 and 270 nm. Disulfide bonds absorb weakly in the absorption range of the aromatic amino acids, mainly at 280nm, and extend thereafter to the 310-330 nm region. Protein samples can be treated with mild reducing agents such as dithiothreitol, 2-mercaptoethanol, or tris-(2-carboxyethyl)-phosphine hydrochloride in order to minimize the absorption of their disulfide bonds. The absorption of the aromatic amino acids is largely dependent on the pH of the sample, e.g., Tyr absorption peak at 275nm (ϵ~1400 M-1cm-1) at neutral pH is red-shifted to 290nm (ϵ~2300 $M^{-1}cm^{-1}$) at alkaline pH due to the production of tyrosinate anion radicals (Tyr-O●−) (Creed, 1984). The absorption at 280 nm differs significantly between proteins depending on their relative contents of Trp, Tyr, Phe, and Cys disulfide bonds. The average molar absorptivities of the chromophores Trp, Tyr, Phe, and Cys of folded proteins in aqueous solutions at pH 7 and 280 nm were found to be 5600, 1490, 200, and 125 $LM^{-1}cm^{-1}$, respectively. Thus,

$Absorbance_{280} = 5600\,[Trp] + 1490\,[Tyr] + 200\,[Phe] + 125\,[S\text{-}S]$

The total absorbance, as the linear combination of the absorbances of these amino acid residues, can be obtained within an accuracy of about +5% (Kelly, Jess, & Price, 2005). The suitability of this method for protein analysis depends on the contents of the responsive amino acids and their relevance to the studied phenomena.

Proteins can also be measured based on the flourescence of the above amino acids. Among these, Trp is the most common, followed by Try, while Phe contributes very little due to poor absorptivity and low quantum yield (Ghisaidoobe & Chung, 2014). Although Trp has a quantum yield similar to Tyr, the indole group of Trp is believed to serves as the principal source of its excitation at around 280 nm and emission at around 350 nm (Teale & Weber, 1957). In the majority of natural proteins, Tyr emission is often suppressed either through energy transfer to Trp or by contact with the peptide chain (Lakowicz, 2013). Compared to the complex characteristics of Trp, Tyr can be regarded as a straightforward fluorophore. Trp is highly sensitive to its surroundings and display two distinct fluorescence lifetimes (0.5 and 3.1 ns) (Gudgin, Lopez-Delgado & Ware, 1981; Swaminathan, Krishnamoorthy & Periasamy, 1994) that are intrinsic to its structure and are unaffected by the excitation wavelength (Albani, 2014a, 2014b). Hydrogen bonding, along with other non-covalent interactions and the polarity of the microenvironment influence the fluorescence maximum and intensity of Trp emission (Gryczynski et al., 1988; Piston & Kremers, 2007).

Spectrophotometric methods can also be used in protein quantification through the measurement of colored dyes formed by the reaction of proteins with different reagents. The Biuret, Bicinchoninic acid, modified Lowry, and Bradford are the most commonly used tests (Table 1). The reaction of peptide bonds with copper (II) ions in an alkaline solution and the formation of mauve-colored coordination complexes between the Cu^{2+} ions and the C—N and C=O atoms on the peptide group (λ_{max} 540 nm) forms the basis of Biuret (or Piotrowski's) test (Chang & Zhang, 2017). Though, the assay is not affected by free amino

acids, it is not suitable for protein samples purified by ammonium sulfate precipitation because buffers, such as Tris and ammonia, interfere with the assay. Several variants of the test such as the bicinchoninic acid (BCA) and the modified Lowry tests have been developed. The BCA test is based on the formation of a deep purple water-soluble complex between Cu^{+} and BCA (λ_{max} 562 nm) (Smith et al., 1985). The BCA/copper complex absorbance is much stronger than the peptide/copper complex in the original Biuret assay, increasing its sensitivity by a factor of *ca* 100 and allowing quantification of protein concentrations in the range 0.0005-2 mg/mL. The presence of up to 5% surfactants in samples is compatible with the BCA assay, which provides an advantage in favor of this assay. In the Lowry assay, Cu^{+} is oxidized to Cu^{2+} by Mo^{VI} in the Folin–Ciocalteu reagent, which is reduced to molybdenum blue (Mo^{IV}). The presence of tyrosine residues increases the sensitivity of the assay to protein concentrations in the range of 0.005-2 mg/mL by contributing to the formation of molybdenum blue (Lowry et al., 1951). The signal can be further amplified by certain organic dyes, such as malachite green and Auramine O, which bind to molybdenum blue (Sargent, 1987).

The absorbance shift of the Coomassie Brilliant Blue G-250 dye forms the basis for fast, sensitive, and precise Bradford protein assay. In an acidic environment, the free cationic form of the dye is red (λ_{max} 465 nm), and it changes to a blue color (λ_{max} 595 nm) when it attaches to a protein (**Figure 2**). The color change is caused by strong non-covalent interactions between the reagent and the protein, including van der Waals forces that cause complexes to form with the carboxyl groups of the protein, electrostatic interactions with the amino groups, and hydrophobic interactions with the aromatic residues. During complex formation, the initially red Coomassie dye donates its free electron to the ionizable groups of proteins, thus altering the proteins' native states and exposing their hydrophobic pockets to bind with the Coomassie dye and form stable blue complexes that can be stored at room

temperature for up to two weeks. The Bradford protein test is characterized as simple, rapid, sensitive, and cost-effective. The Bradford test is not influenced by interference from minerals or carbohydrates present in the samples, however, the presence of detergent sodium dodecyl sulfate (SDS), which is used for protein denaturation in SDS-PAGE can affect the results. When proteins are present at concentrations below their critical micelle concentration (CMC), SDS prevents dye binding leading to an underestimation of protein concentrations. At concentrations above the CMC, SDS becomes highly linked with the dye leading to an overestimation of the protein content. However, the inclusion of cyclodextrins in the test mixture may help eliminate these problems. Furthermore, the utilization of a high buffer concentration during sample preparation may lead to an overestimation of the protein concentrations due to the formation of a conjugate base with the reagent. The protein sequence and its similarity to the reference protein are crucial factors in the Bradford test because the reactions of proteins differ based on the dye's affinity for arginine and lysine residues (Georgiou et al., 2008). Comparing the different spectrophotometric methods for the determination of total proteins in samples of bovine milk powder, the results of the Bradford technique were found to be the most similar to the Kjeldhal method (Kamizake et al., 2002). Dye-based spectrophotometric methods are specially more sensitive and require less samples than the titremetic methods.

Milk and dairy products' proteins are subjected different degrees of proteolysis by microbial and non-microbial enzymes. A fast and convenient spectrophotometric assay utilizing the O-phthalaldehyde (OPA) is used for measuring the degree of protein hydrolysis at 340 nm. The OPA assay relies on the interaction between OPA with free amino groups that become available when a protein substrate undergoes proteolysis (Church et al., 1985). This method is commonly used to evaluate the extent of proteolysis in yogurt (Li et al., 2023) and cheeses (Myagkonosov et al., 2020).

2.3. Other Methods

The Dumas method relies on burning the sample in oxygen to generate carbon dioxide, nitrogen oxides, sulfur dioxide, and water. These gases are collected in a specific sorption column that utilizes heated $PbCrO_4$, Cu, NaOH, or P_4O_{10} to remove the appropriate amounts of SO_2, O_2, CO_2, and water, while simultaneously measuring NO_2 using a thermal conductivity detector. The Dumas method has been shown to provide results equivalent to those of the Kjeldahl method in various applications suggesting possible substitution of the Kjeldahl method in certain situations (Wiles et al., 1998, Simonne et al., 1999, Owusu-Apenten, 2002).

Calorimetric methods can also be used, where the analyte is held in an electrolytic chamber while a known amount of electricity is used to neutralize, oxidize, reduce, or precipitate the material, and analyze it (Tailor & Smith, 1959). The formula Q=nzF, where Q is the total electrical charge, n is moles of oxidized peptides, z is the number of electrons transferred per molecule during the redox process, and F is the Faraday constant (9.65×10^4 C/mol). Tryptophan, cysteine, tyrosine, and methionine are the four biological amino acids that may be oxidized; tryptophan has a redox potential of 0.23, cysteine of 0.93, tyrosine of 1.02, and methionine of 1.5 (Zhao, Zare & Chen, 2019). The method of identifying and determining the sulfhydryl and disulfide groups in proteins by calorimetric titration using excess silver (I) is able to quantify small amounts of proteins (20-1000 µg/mL) with an accuracy of within 4% (Ladenson & Purdy, 1973).

3. Chromatographic Techniques for the Separation and Purification of Proteins

Chromatographic methods offer several advantages in protein analysis by allowing separation and quantification of individual proteins and peptides in samples. Different

techniques utilize differential properties of the protein, e.g. polarity, hydrophobicity, ionic charges, size, and selective affinities, to perform the separations as explained below.

3.1. Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

RP-HPLC is a very useful technique for the separation and quantification of milk proteins (Mitulović & Mechtler, 2006). Milk samples need to be skimmed (e.g. by centrifugation at 3000 g at 15 °C for 30 min), clarified (e.g. by the addition of 7 volumes of 0.1 M Bis-Tris buffer (pH 8.0) containing 8 M urea, 1.3% trisodium citrate), and reduced with 0.3% dithiothreitol (DTT) overnight at 4 °C (Miranda et al., 2004). In one method, a milk sample (200 µL) was mixed with 3780 µL of dissociating buffer (7 M urea and 20 mM Bis-tris propane) and then 20 µL/mL of mercaptoethanol was added and the sample was filtered through a 0.22 or 0.45 µm PVDF filter (Paludetti et al., 2018). It was suggested that diluting milk samples in an optimized mixture of acetonitrile: water: trifluoroacetic acid (340:660:1, v/v/v) during sample preparation enables optimal wetting, solubility, and hydrophilicity of the proteins leading to sharper peaks and improved separation (Yüksel & Erdem, 2010).

Milk protein fractions and genetic variants can be separated in one run with very high resolutions using RP-HPLC methods (Jensen et al., 2012). Protein separations were carried out using C4, C5, C8, and C18 reversed-phase columns **(Table 2)** and a clear separation on a C18 column is shown in **Figure 3**. In a recent study, RP-HPLC was used with traditional SDS-PAGE, cDNA sequencing, and advanced proteomic MS/MS techniques to evaluate the molecular diversity of camel milk (Ryskaliyeva et al., 2018). Complete separation of casein macropeptide, proteose-peptones, α-lactalbumin (overlapped glycated forms), bovine serum albumin, and β-lactoglobulins A and B was obtained within 6 min on a C8 column (Sturaro et al., 2016). The bis-Tris/citrate buffer (pH 7) with guanidine hydrochloride (instead of urea), DDT (instead of 2-mercaptoethanol), and a sophisticated seven steps-gradient was used to

achieve simultaneous separation and quantification of caseins and whey proteins in less than 40 min on a C8 stationary phase at 45 °C (Bonfatti et al., 2008). For example, successful separations using RP-HPLC were obtained for the milk caseins of water buffalo (Bonfatti et al., 2013) and mare (Fessas et al., 2001). Age gelation of UHT milk was also studied using RP-HPLC coupled with mass spectrometry (MS/MS) (Reynes et al., 2018). Some limitations may still remain, e.g., "noisy peaks" are induced in the κ-/αs-2 casein region due to plasmin activity in milk leading to partial co-elution of γ-casein fragments with β-casein A1 or A2 (Bonfatti et al., 2008). Similarly, a RP-HPLC octadecyl-silica monolithic column was used to separate the major bovine casein proteins within five minutes (Ramírez-Palomino et al., 2014). However, Vincent et al. (2016) suggested different RP-HPLC and MS parameters optimization to overcome the co-elution of bovine milk proteins and to identify the differences in protein variants in bulk milk samples.

3.2. Hydrophobic Interaction Chromatography (HIC)

HIC is based on the adsorption of hydrophobic regions of proteins on immobilized hydrophobic ligands, such as butyl, octyl, and phenyl groups, linked to the column in the presence of high salt concentrations in the mobile phase (Rustandi, 2013). To enhance their interactions with the stationary phase, protein samples are initially added to HIC resin in high ionic strength buffer. Subsequently, the proteins are eluted in descending order of hydrophobicity by reducing the ionic strength of the buffer (Flatt, 2019). The chromatographic resolution is influenced by the hydrophobic ligand types and salt concentration in the solution (Zhang et al., 2010). Protein charges might affect proteins' retention and separation, e.g., uncharged histidine residues in lysozyme increased retention while charged histidine residues decreased it (Fausnaugh & Regnier, 1986). The HIC elution profiles of the casein fractions can be utilized to identify the presence of different milk types (goat, ewe, and bovine) in mixtures (Bramanti et al., 2003). HIC elution with a salt gradient

was also employed to successfully recover β-lactoglobulin from whey protein concentrates, with a recovery of 45.2% (Santos et al., 2011).

3.3. Ion Exchange Chromatography (IEC)

IEC separates proteins according to their charge properties based on the attraction of oppositely charged molecules by the stationary phase (Cummins et al., 2017). Two IEC modes, i.e. cation- and anion-exchange, are enabled by the presence of a stationary phase, which is an inert organic matrix chemically derivatized with ionizable functional groups that hold displaceable oppositely charged counter ions, and an aqueous buffer mobile phase (Cummins, Dowling & O'Connor, 2011). Proteins adsorb on the resins according to their charge-to-mass ratio and can be desorbed by changing the buffer's pH and ionic strength (Ali et al., 2010). A wide range of ion-exchange chromatography methods have been used for the analysis of whey proteins (Goodall et al., 2008; Voswinkel & Kulozik, 2011; Santos et al., 2012; Dizaji, 2016). Before sample injection into the IEC system, phospholipids were removed to prevent the blocking of the adsorption of proteins (Voswinkel, 2011).

The separation of α-lactalbumin and bovine serum albumin but not β-lactoglobulin variants was effective using anion-exchange chromatography (Ye et al., 2000; Kim et al., 2003). About 60% of bovine β-lactoglobulin was recovered in a pure form in a single step using a Mon Q 550 GL anion-exchange column with a salt gradient elution (Santos et al., 2012). The three major bovine milk proteins' binding strength on an anion exchangers follows the order serum albumin dimer > serum albumin monomer > α-lactalbumin (Weinbrenner & Etzel, 1994). The displacement of the negatively charged α-lactalbumin by β-lactoglobulin from an anion exchange resin or anion membrane has been reported in some cases (Goodall et al., 2008).

Cation exchange chromatography was successfully used to separate bovine casein fractions (Hollar et al. 1991). DEAE-cellulose ion exchange chromatography and SDS-PAGE

techniques were used to separate and characterize camel milk proteins and differentiate them from bovine milk proteins (Saliha et al., 2013). Sheep's milk αs-casein was effectively purified and separated using DEAE-cellulose ion exchange and sephadex-G-75 size exclusion chromatography (AlKhalidy & Dosh, 2023). DEAE ion exchange and gel filtration methods are used to separate bovine β-lactoglobulin A, B, and, α-lactalbumin in bovine milk (Yoshida, 1990) while DEAE-sepharose fast flow anion exchange chromatography was employed to isolate and purify whey proteins (Mao et al., 2016). The IEC and two-dimensional gel-based proteomic approaches were used to separate bovine whey protein isoform patterns and several new minor whey proteins were identified (Fong et al., 2008). IEC has also been used to determine the amino acids present in cheese (Hogenboom et al., 2017).

3.4. Size-Exclusion Chromatography (SEC)

In SEC, also referred to as gel permeation or gel filtration chromatography, proteins are separated on porous gels according to their sizes and molecular weights using adequate calibrations. SEC can be used to detect and quantify aggregation of protein or modified proteins having different quaternary structures (Held & Hofe, 2017). SEC analyses of raw and heat-treated bovine and donkey milk proteins provided compatible results with RP-HPLC and revealed more information about heat-aggregated caseins in both milks (Pinho, 2012). SEC was successful in separating casein hydrolysates according to the mean length of the peptides (Silvestre et al., 1994). A comparison between SEC and asymmetrical flow field-flow fractionation technique provided good separation of low molecular weight components (Kang et al., 2011).

3.5. Affinity Chromatography

Specific biological-like interactions, such as antigen-antibody binding, are utilized in affinity chromatography to separate specific proteins (Hage, 1999). In this technique, the process consists of three main steps: incubation of the sample with an immobilized specific antibody ligand to allow binding, washing away the other non-specific components, and finally elution of the target protein in a suitable buffer (Anusha & Sirisha, 2018). Lectin affinity chromatography has been used in the purification of glycoproteins due to lectin's ability to recognize and bind certain types of carbohydrate residues (Flatt, 2019). The purification is made easy by tagging a known sequence ranging from a short fragment of amino acids to the whole domain or the whole protein of interest. Tags facilitate protein purification by acting as markers of protein expression (Anusha & Sirisha, 2018). In this process, untagged proteins will pass through the column first, and the target protein(s) are then eluted with a suitable eluent that competes with the tag for binding of the protein or by decreasing pH and the affinity of the tag for the resin (Flatt, 2019). Sheep milk caseins were separated into four fractions (α-s1, α-s2, β, and κ) using affinity chromatography attached with activated thiol-sepharose 4B and it was concluded that this result can be used to determine the polymorphism in asein fractions (Dall'olio, 1990).

3.6. Fast Protein Liquid Chromatography (FPLC)

Separation and purification of proteins in their native state have been achieved by FPLC (Madadlou, O'Sullivan & Sheehan, 2011). The possibility of protein denaturation is decreased by the comparatively low back pressure needed to drive the high flow rates (Gonzalez-Llano et al., 1990). Mostly, cross-linked agarose beads with a variety of sizes and surface ligands serves as the stationary phase in FPLC. FPLC can be used for the separation of proteins on ion exchange, SEC, reversed-phase, and affinity chromatographic columns but the most commonly used are anion exchange columns (Madadlou et al., 2011). Usually, UV

detectors are used to measure the protein concentration at an absorption wavelength of 280 nm (Flatt, 2019).

FPLC has been used to purify α-, β-, and κ-caseins for further investigations (Plank et al., 2008). Good separation and quantification of the major milk caseins were accomplished using anion-exchange FPLC on a Mono Q column at pH 7 (Davies & Law, 1987). Cation-exchange FCLP was used for separating major casein fractions (Hollar et al., 1991) while anion-exchange FCLP was used to separate α-lactalbumin and β-lactoglobulin from whey (Geng et al., 2015). The use of FPLC with RP-HPLC for the fractionation of soluble nitrogen fraction of fermented milk containing angiotensin-I-converting-enzyme (ACE) inhibitory peptides yielded promising results (Gobbetti et al., 2000).

4. Electrophoretic Techniques for Protein Separation, Visualization, and Identification

Electrophoretic techniques separate proteins based on their sizes and charges and are very reliable in providing finger-prints of protein compositions. They can be used for semi-quantitative analysis, e.g. by densitometry of the separated bands, as well as for quantitative analysis by capillary electrophoresis.

4.1. One Dimensional Gel Electrophoresis

Gel electrophoresis is a simple method that is commonly used for visual phenotyping of proteins on different types of gels (Ribadeau-Dumas et al., 1989). Gel electrophoresis methods are convenient, fast, inexpensive, and require only micrograms quantities of protein. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) does not require prior fractionation of whey and caseins and is used for simultaneously separation of all milk proteins according to their molecular weight (Basch et al., 1985). Differentiation of whey proteins and separation of bovine caseins into four different bands representing α_{s1}-, α_{s2}-, β-

and κ-caseins can be achieved using SDS-PAGE and a reducing agent (**Figure 4**) (Yelubaeva et al., 2017).

SDS-PAGE has been successfully used for the proteomic profiling of heat-treated milk proteins (Felfoul et al., 2017) and for the analysis of milk proteins in cheese after dissolving samples in 8 M Urea (Hailu et al., 2018; Mbye et al., 2021). The inclusion of urea in separator gel significantly improves the separation of caseins with no deleterious effect on whey protein migration. Urea-PAGE electrophoresis denatures proteins without neutralizing their charges, thus allowing their separation according to their charge-to-mass ratio rather than on sole basis of molecular weight. Proteins having similar electrophoretic mobility in SDS-PAGE may have different mobilities in urea-PAGE depending on their intrinsic charges and the gel's pH. In the presence of reducing agents, such as 2-mercaptoethanol or dithiothreitol, together with urea, casein separations can be carried out at both alkaline and acidic pHs. On the other hand, whey protein separations are carried out at alkaline pHs without these reagents (Ribadeau-Dumas & Grappin, 1989). While whey protein was separated optimally on a 12% polyacrylamide gel in the absence of urea and with a top layer of large pore gel, caseins separated best on 8% polyacrylamide gel with 4 M urea. The reducing agents break up the intermolecular disulfide bridges while urea facilitates the dissociation of the intermolecular casein micellar aggregates (Ng-Kwai-Hang & Kroeker, 1984).

Gel electrophoresis has been used to study the quantities of distinct proteins in milk from various animal species, as well as the impact of genetic and environmental variables, and various processing techniques (Felfoul et al., 2017; Sharma et al., 2017). Such studies usually involved large numbers of samples, and multiple samples can be run in parallel to provide easily interpreted qualitative results (Ng-Kwai-Hang & Kroeker, 1984). The electrophoretic patterns of bovine and goat samples clearly indicated the presence of two

whey proteins, β-lactoglobulin and α-lactalbumin, as well as the four casein variants: α_{s1}-, α_{s2}-, β-, and κ-caseins. Infants' milk protein allergies are often triggered by β-lactoglobulin and α_{s1}-casein, which are not present in human milk proteins (Sharma et al., 2017). Variability in the casein and whey milk proteins of bovine, goat, sheep, buffalo, and camels were shown by gel electrophoresis (Yasmin et al. 2020). The determination of protein content of skim milk (semi-quantitative), non-fat dry milk, total protein concentrate, and whey protein concentrate were performed by gel electrophoresis and were followed by scanning densitometry. This required proper calibration due to variability in the binding capacities for anionic dyes depending on the contents of basic amino acids in the proteins (Basch et al., 1985).

4.2. Two-Dimensional Gel Electrophoresis (2-DE)

Complex protein mixtures can be separated on SDS-PAGE using two-dimensional gel electrophoresis (2-DE) frequently using reducing substances (Chevalier et al., 2009). This technique relies on two molecular characteristics: isoelectric focusing (IEF), which separates proteins based on their charge and molecular weight, which separates proteins based on their size (**Figure 5**) (O'donnell et al., 2004). An alternative application, provided by non-reducing PAGE, permits the imaging of disulfide-linked complexes such as those present in pasteurized and heat-treated milk samples. 2-DE has been employed to explore proteolysis of β- and κ-caseins (O'Donnell et al., 2004). β-Casein proteolytic fragments, ranging in size from 9 kDa to large fragments just a few amino acids below the mass of β-casein to, have been separated on 2-DE gels (Yamada et al., 2002). In bovine whey, C-terminally shortened forms of β-lactoglobulin have also been identified using the 2-DE (Zappacosta et al., 1998).

Researchers have studied the separation of bovine milk protein components using 2-DE coupled with different mass spectrometry techniques (Galvani, Hamdan & Righetti, 2001). 2-DE was used successfully to separate and identify isoforms of κ-casein having

different phosphorylation and glycosylation patterns (Claverol et al., 2003), and different casein isoforms in bovine (Holland et al., 2006), donkey (Cunsolo et al., 2009), and goat milks (Roncada et al., 2002). 2-DE linked to Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometry was employed to examine the whey proteins in healthy and mastitis-infected animals (Alonso-Fauste et al., 2012), identify allergenic milk proteins in two goat breeds (Mansor et al., 2020), discover known and novel proteins in bovine whey (Lee et al., 2018), and profile the variation in αs1-casein, κ-casein and β-lactoglobulin in different Bulgarian cattle breeds (Zagorchev et al., 2013). Milk proteins from six goat breeds were separated by 2-DE and reported 25 to 102 spots in the different breeds (Kumar et al., 2013).

By labelling each extract with spectrally resolvable, size- and charge-matched fluorescent dyes known as CyDye DIGE fluorophores, multiple protein extracts can be separated using two-dimensional differential gel electrophoresis (2D-DIGE) This technique is used to study milk fat globule proteomes from control- and *Mycoplasma agalactiae*- infected sheep milks that were labeled with N-hydroxysuccinimidyl ester of cyanine dyes Cy3 and Cy2, respectively (**Figure 6**) (Addis et al., 2011).

4.3. Isoelectric focusing (IEF)

IEF separates proteins and peptides according to their charge as defined by the pK_a values of the molecule (Friedman et al., 2009). Milk protein components were separated using the IEF technique in 1-mm thick polyacrylamide gels containing ampholytes, 7 M urea, and 0.1% 2-mercaptoethanol. IEF was employed for phenotyping milk proteins on ultra-thin-layer polyacrylamide gels in a single run (Seibert et al., 1985). IEF was used to separate and identify α_{s1}-casin A, B, C; α_{s2}-casein B; β-casein A^1, A^2, A^3, B, C, κ-casein A, B, α-lactalbumin B; and β-lactoglobulins A, B, C (Trieu-Cuot & Gripon, 1981). IEF followed by PAGE was used to compare milk proteins of bovine, goat, pig, mouse, and human and found

that bovine and caprine milk proteins show great similarity while non-ruminant milk proteins showed least similarity (Kim & Jmenez-flores, 1993). A reference method based on IEF was developed to trace the presence of bovine milk in cheeses made from goat and ewe milks (Spolijaric et al., 2013).

4.4 Western Blotting

Western blotting involves transferring or blotting single proteins from electrophoresis separation gels to membranes where they can be visualized. Several steps are involved including sample preparation, 1D or 2D gel electrophoresis, blotting to the membrane, and antibody binding, detection, imaging, and analysis. Western blotting has been used to identify and quantify differentially expressed bovine and buffalo milk proteins marking the transition from subclinical to clinical mastitis and found cyclase associated protein to be up-regulated in sub-clinical mastitis in both milks (Maity, Das & Ambatipudi, 2020). Analysis of immunologically-active peptides and allergic proteins have investigated using Western blotting techniques (Picariello et al., 2019). Moreover, Western blotting was used to analyze milk protein synthesis and regulatory pathways such as the activity of the mTOR pathway and β-casein expression, the effects of individual essential amino acids on cellular signaling, and fractional protein synthesis rates in bovine mammary cells (Appuhamy et al., 2011). Western blotting was also successfully applied to checking goat, ewe, and buffalo cheeses adulteration with bovine milk (Molina et al., 1995).

4.5 Capillary Electrophoresis (CE)

In electrochemical processes, electric currents affect how molecules with an electric charge move through a capillary column. This enables simultaneous high-resolution isolation of casein and whey, provides accurate quantification, and offers an excellent opportunity to identify genetic variations, phosphorylation, and glycosylation patterns in milk proteins **(Figure 7)** (Heck et al., 2008). The separation of lactoferrin by CE is poor compared to other

whey proteins because of its large size (80 kDa), high basic isoelectric point (pI, 8-9), and low concentrations (Riechel et al., 1998; Indyk et al., 2007). However, it was a possible to detect lactoferrin in infant formulas by using a rather complex running buffer in a separation time of 30 minutes (Li et al., 2011). CE has been used to separate camel milk caseins and α-lactalbumin (Mohamed et al., 2020) and to investigate buffalo milk adulteration by bovine milk by exploring α-lactoglobulin as a marker of adulteration (Trimboli et al., 2019). Genetic polymorphism of Australian sheep breeds was studied by CE and was found to be greatly affected by the milk composition and yield (Clement et al., 2006). Milk, cheese and whey proteins and peptides were also successfully characterized by CE utilizing different buffers (Strickland et al., 2001).

5. Selective and Fast Methods for Determination of Milk Proteins

5.1. Infra-Red Spectroscopy with Chemometrics

Currently, rapid, non-destructive, and cost-effective infra-red (IR) spectrophotometric methods are used for routine milk protein analysis. IR spectroscopy coupled with multivariate chemometric analysis is used to develop prediction models that are appropriate for the determination of milk components including proteins (Dufour, 2011). Calibration models, using 100-150 samples that are previously analyzed by a reference method (training or calibration set), are developed to relate the spectral and reference data through regression analysis. Another set of 100-150 samples will also be analyzed by IR and the reference method and used to validate the model (Yakubu, 2022).

The IR absorption spectra are based on different vibration modes of molecular chemical bonds, *e.g*. stretching, bending, *etc*. Transmission IR spectroscopy detects vibrations of covalent bonds in the near infrared (NIR, 800-2500 nm) or mid infrared (MIR, 2,500-25,000 nm) regions (Mohamed et al., 2020). In NIR, protein-specific vibrations occur

at the following wavelength: amide B 1640 -1670 nm (first overtone of NH stretching vibration), amide A and amide I bands at 2056 nm and 2180 nm (C-H combination). In MIR, vibrations occur at the following bands: amide I band 6060 nm (80% C=O stretch, 10% C-N stretching and 10% N-H bending vibrations), amide II band 6500 nm (60% N-H and 40 % C-N stretching vibration), and amide III band 8064 nm (30% C-N stretching, 30% N-H bending, 10% C=O stretching, 10% O-C-N bending vibrations). Scattering of light by protein micelles in visible and short-wavelength NIR (400-1100 nm) can also successfully be used for the quantification of total proteins in milk (Bogomolov & Melenteva, 2013). NIR and MIR absorption wavelengths are used to describe the CH, C=O, and peptide linkages in milk proteins as well as the -OH groups in fatty acids, esters, amino acids, and lactose.

In comparison with NIR spectroscopy, MIR was claimed to produce more precise results for total protein content in milk powders (Wu et al., 2008). MIR was used to determine the casein composition (α-, β-, and κ-casein) of goat milk (Daniloski et al., 2022) and a NIR approach was utilized to determine the lactoferrin content in bovine milk (Soyeurt et al., 2020). Raw bovine milk proteins were analyzed using MIR and attenuated total reflectance spectroscopy and it was found that this method has the potential to give rapid results (Etzion et al., 2004). Several companies have developed commercially available equipment with calibrations for milk analysis that are widely used by the dairy industry. Limitations of IR spectroscopy are caused by the light scattering and specular reflection by fat globules (Holyrod, 2013; Yakubu et al., 2022) and by the overlap of the strong bands of water with protein bands (Aernouts et al., 2011, Mohamed et al., 2020).

5.2. Enzyme-Linked Immuno-Sorbent (ELISA) Methods

ELISA methods are based on the binding of antibodies and antigens with high specificity and affinity (Hsieh, 2010). Antigen (protein) reaction-based ELISA tests use enzyme-labelled antibodies to enable quantitative measurement of the complexes using UV-

visible spectrophotometers (Sakamoto et al., 2018). Many commercial ELISA kits are available for the analysis of different milk proteins with variability in terms of their sensitivity, specificity, reporting units, and format (Downs & Taylor, 2010). The assay's specificity is determined by the efficiency of the binding and by cross-reactivity with other proteins. Immunological methods, using polyclonal antisera, are available for the quantification of α-casein, β-casein, and β-lactoglobulins (Levieux & Venien, 1994; Anguita et al., 1996; Haza et al., 1999). Adulteration of goat, sheep, or buffalo milk with bovine milk was detected using highly selective ELISA employing a monoclonal antibody that identifies a species-specific epitope on the heavy chain of bovine IgG1 and IgG2 (Hurley et al., 2004). Bovine milk adulteration in yak milk was detected using a specialized ELISA technique that targets bovine αS1-casein (Ren et al., 2014).

Another class of immunoassays often employed in the study of milk proteins is radial immune-diffusion (RID) assays. In this technique, an antibody is added to a hot medium composed of agar or agarose dissolved in buffered normal saline and the mixture is placed onto a petri dish and allowed to harden. The antigen (protein) solution is poured into a gel well and as it diffuses radially, a circle of precipitin is formed in the media and its diameter is used for quantification with reference to diameters of circles created by known concentrations of the antigen (Stanley, 2002). RID methods have been used to determine lactoferrin content in healthy and subclinical mastitis cows (Hagiwara et al., 2003), lactoferrin and immunoglobulin G in bovine, buffalo, and camel milks and the effect of heat treatment on their stability (Elagamy et al. (2000), and the variability of bioactive proteins such as lactoferrin, immunoglobulin G using an ELISA kit specifically created for camel milk (Mohamed et al., 2021).

6. Structural Characterization of Milk Proteins

6.1. X-Ray Crystallography

X-ray crystallography can be used to obtain structural information for separated proteins, specifically three-dimensional configuration by employing X-ray diffraction of the protein's crystalline form (Flatt, 2019; Sawyer et al., 2002). Before the X-ray examination, the non-globular casein molecules are allowed to form crystallizable complexes with other substances (Holt & Sawyer, 1993). For instance, a study using X-ray crystallography on β-lactoglobulin revealed the three structural variations A, B, and C (Bewley et al., 1997). X-ray crystallography was also used to study the effect of pH on the structural differences and the crystalline behavior of proteins (Timasheff & Townend, 1964).

6.2. Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR of proteins is used to study the chemical structure and dynamics of isolated proteins, nucleic acids, and their complexes (Wahlgren & Drakenberg, 1995), e.g. the characterization of structural changes in caseins (Humphrey & Jolley, 1982), casein micelles (Kakalis, Kumosinski & Farrell, 1990), whey proteins (Lübke et al., 2002), proteins and phosphorylated proteins (Belloque & Ramos, 2002). NMR analysis of 10% milk powder in D_2O allowed for easier locking and increased resolution (Hu et al., 2004). ^{1}HNMR spectroscopy was used to compare the behavior of β-lactoglobulin under pressure and showed that the core of the β-lactoglobulin variant A was unfolded faster than variant B (Belloque & Ramos, 1999). When heated during milk processing, β-lactoglobulin interacts hydrophilically with specific phospholipids, thereby affecting their tertiary structure and function. This information is based on data obtained from diffusion NMR studies and circular dichroism (Kasinos et al., 2013). ^{1}H-NMR combined with deuterium exchange reactions was used to monitor the unfolding of whey proteins induced by heating, pressure, and foaming (Tanaka & Kunugi, 1996; Belloque & Smith, 1998). To obtain further information on the

conformational changes that take place during protein unfolding, deuteration is combined with 2D-^{1}H NMR (Belloque & Smith, 1998).

Among the various NMR spectroscopic techniques used to study milk proteins, ^{31}P NMR spectroscopy is very useful in studying caseins and whey proteins (Belloque & Ramos, 1999). Casein aggregation behavior after processing and under various environmental conditions have been explored using ^{31}P NMR, e.g. increases in inorganic phosphate mobility with decreasing calcium (Belton et al., 1985), and assessment of the degree of chelation with calcium and casein mobility in the matrix (Rulliere et al., 2013).

6.3. Mass Spectrometry and Proteomic Investigations

Proteomics is an effective tool for the characterization of milk and dairy products due on basis of their proteins and peptides (Agreagan et al., 2021).

Common proteomic workflows used in food authentication are illustrated in **Figure 8**. These techniques are crucial because they facilitate the investigation of several milk proteins at once and enabling their detection, characterization, and identification (O'Donnell et al., 2004). Complicated experimental designs are needed for proteomic studies aiming at characterizing post translational modifications of proteins. In such scenarios, mass spectrometry (MS) enables the elucidation of protein complexes, their components, and functional connections, as well as the global measurements of proteins (Flatt, 2019). Interpretation of the generated mass spectra is aided by bio-informatic tools and mass spectral libraries of proteins and peptides.

Proteomic analyses employ sophisticated analytical techniques to gain multi-dimensional information about protein and peptide structures and/or functions. Combination of one or two separation techniques (electrophoresis, HPLC, ion-exchange, or size-exclusion, affinity chromatography, and/or immuno-precipitation) with tandem mass spectrometry (MS/MS), MALDI-TOF mass spectrometry, and/or cDNA sequencing can be used (Issaq et

al., 2005; Tang et al., 2008; Liu et al., 2010; Mitulović, 2015). Cataloguing protein expression, describing protein interactions, and locating protein modification sites are the three main uses of MS in proteomics (Han et al., 2008). Several studies have investigated the potential applications of milk proteomics in general along with the modifications that milk proteins undergo during lactation, processing, and storage (O'donnell et al., 2004; Cunsolo et al., 2011b; Abd El-Salam, 2014).

Protein expression, structure, genetic sequence variations, phosphorylation level, and post-translational changes, as well as changes driven by the environment, processing, or storage, can all be analyzed by milk proteomics (Manso et al., 2005). Human milk proteins were analyzed using anion exchange chromatography and mass spectrometry (Mange et al., 2008). Low-abundance proteins in the whey fraction of human milk (enriched by ProteoMiner beads) were identified over a 12-month lactation period by employing liquid chromatography-tandem mass spectrometry (LC−MS/MS) (Liao et al., 2011). Tandem mass spectrometry (MS/MS) approaches were applied to bovine milk κ-caseins to pursue and confirm post-translational modifications, including phosphorylation, glycosylation, and disulfide linkages (Holland et al., 2004, 2005). Comparative proteomics were employed to assess milk adulterations and allergenicity of sheep, camel, buffalo, horse, and donkey milks (D'auria et al., 2005; Hinz et al., 2012). Data from previous investigations was merged to compile an exhaustive list of 573 non redundant annotated bovine protein entries, most of which were categorized under pathways and the immune system response (D'Alessandro et al., 2011).

Proteomics have been used to study bovine α- and β-caseins as model phosphoproteins (Zhou et al., 2006; Wu et al., 2007). The lactosylation sites of β-lactoglobulin were identified using reversed-phase liquid chromatography-electrospray ionization (ESI) analysis (Leonil et al., 1997). Lactosylation studies on β-lactoglobulin using

reducing sugars showed that lysine 100 is the most engaged amino acids followed by lysine 47 (Fogliano et al., 1998). However, in dry state, active amine groups in proteins are bound by lactose instead of lysine 101. Initially, lactose was found to attach to lysines 47 and 91 of β-lactoglobulin (Morgan et al., 1998). In heated milks (e.g. evaporated milk), lysines 47 and 60 might be identified, while lysines 60, 75, 77, 83, and 135 were more preferentially associated with lactose in milk sample subjected to mild heating. It was evident from this research that milk samples with different temperature histories exhibit different lactose binding sites on lysine Meltretter et al. (2014).

6.3.1. Top-down Approaches

In top-down approach, intact protein ions are fractionated, separated, and analyzed by ESI-MS for identification and quantification leading to greater amino acid sequence coverage (McLafferty et al., 2007). Detection of existing modifications can readily be achieved via top-down proteomics, simultaneously in one spectrum. The top-down analysis of intact proteins preserves the labile structures of a protein that are destroyed in the bottom-up analysis and, as such, it is a superior method for examining protein modification (Siuti & Kelleher, 2007). Top-down proteomics further highlighted variations in major milk isoform and proteo-forms across bovine breeds. Notably, α-lactalbumin, all phosphorylated forms of α-S2-casein A, β-casein A2, and κ-casein B-1P accumulating more in Jersey than in Holstein-Friesian milk (Vincent et al., 2016; Vincent et al., 2018). Milk shelf-life was investigated using a top-down approach to identify proteolytic processes leading to age gelation of UHT milk over time. It was found that UHT treatment produced lactosylated proteo-forms and that the abundance of intact native proteins decreased over storage time while their degradation products increased (Raynes et al., 2018). However, top-down proteomics analysis is still undergoing development as it is facing many technical challenges such as protein solubility, proteome

complexity, dynamic range of the proteome, data analysis, and linking proteo-forms to biological function (Melby, 2021).

6.3.2. Bottom-up Approaches

In bottom-up or shotgun approaches, protein mixtures or purified proteins are digested by enzymes to generate peptides that are separated by chromatography or by electrophoresis and analyzed by mass spectrometry to identify the resulting hydrolytic peptides. Usually, a limited number of separated peptides can be identified by shotgun proteomics, leading to partial coverage of protein sequences (Steen & Mann, 2004). Electrospray ionization (ESI) and matrix-assisted laser desorption (MALDI) techniques were used in parallel to confidently identify of 39 bovine milk proteins (Mollé et al., 2009). MALDI was employed for the identification of smaller, more basic peptides, while ESI was favored for larger, more hydrophobic peptides. As a result, the proteome coverage acquired from mass spectrometers with various ionic materials and detectors could be used complementarily. Qualitative differences in the proteomes of bovine breeds were explored using a shotgun approach and resulted in the identification of 186 milk proteins, including not only the most prominent proteins but also many minor ones (Vincent et al., 2015). Bovine milk serum was analyzed using shotgun proteomics, resulting in the identification of 192 proteins (Hettinga et al., 2011).

6.3.3. Multiple Reaction Monitoring (MRM)

Proteins in dairy products have been quantified using MRM technique (Lutter, Parisod & Weymuth, 2011). Some of the applications include the study of lactosylated whey protein fragments of milk powders (Le et al., 2013), post-translational modifications of β-lactoglobulin, and hydrolysis of asparagine in milk and dairy goods caseinates (Meltretter,

Wüst & Pischetsrieder, 2013, 2014), and the study of glycol-oxidation using casein micelles and sodium (Moeckel et al., 2016).

6.3.4. Isobaric tag for relative and absolute quantification (iTRAQ)

Identification and quantification of whey proteins of bovine, buffalo, goat, camel, and yak milks using iTRAQ technique is a reliable method. Whey proteins were digested Using trypsin for 16-18 hours at 37 °C and the resulting peptide concentrations were measured at OD_{280}. Thereupon, peptide mixtures were labeled with iTRAQ reagents following manufacturer instructions and the peptides were separated using strong cation exchange chromatography on a polysulfethyl column. Subsequently, they were identified using RP-HPLC on Zorbax 300SB-C18. Finally, the peptides were evaluated using Q-Exactive mass spectrometry, with the mass range of 300-1800 m/z (Yang et al., 2013).

7. Concluding Remarks

This review discussed and compared different techniques used for protein analysis of milk and dairy products, ranging from titration, spectroscopic, chromatographic, electrophoretic, and electrochemical methods, to advanced mass spectrometry techniques. The suitable analytical approach to be used depends on the objectives of the analysis. Traditional analytical methods are still used in obtaining total protein contents, and for the identification, characterization, and quantification of different proteins in milk and dairy products. Chromatographic techniques (HPLC, HIC, IEC, SEC, and affinity chromatography) and capillary electrophoresis methods can be used to separate and purify proteins. Gel electrophoresis techniques are commonly used in protein separation, visualization, and identification. Isoelectric focusing is a technique based on the fact that proteins have different charges at different pH gradients and leading to the separation of proteins on the gel

according to their isoelectric points. Western blotting techniques, which requires transferring, or blotting, single proteins from electrophoresis separation gels to a nitrocellulose membrane for further analysis. IR methods, immunoassay techniques, such as ELISA and RID, are also available for fast determination of selected proteins. XRF and NMR can be used to identify 3D structures of protein. Bottom-up and top-down proteomics analyses involving chromatography coupled to MS/MS or MALDI-TOF analysis are used in the analysis of protein mixtures. In conclusion, the selection of the appropriate method depends on what the analyst wants to achieve.

CRediT authorship contribution statement

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References

Abd El-Salam, M. H., 2014. Application of proteomics to the areas of milk production, processing and quality control: a review. International Journal of Dairy Technology 67, 153–166. https://doi.org/10.1111/1471-0307.12116

Addis, M. F., Pisanu, S., Ghisaura, S., Pagnozzi, D., Marogna, G., Tanca, A., Biosa, G., Cacciotto, C., Alberti, A., Pittau, M., Roggio, T., Uzzau, S., 2011. Proteomics and pathway analyses of the milk fat globule in sheep naturally infected by Mycoplasma agalactiae provide indications of the in vivo response of the mammary epithelium to bacterial infection. Infection and Immunity 79, 3833–3845. https://doi.org/10.1128/IAI.00040-11

Aernouts, B., Polshin, E., Lammertyn, J., Saeys, W., 2011. Visible and near-infrared spectroscopic analysis of raw milk for cow health monitoring: reflectance or

transmittance? Journal of Dairy Science 94, 5315–5329. https://doi.org/10.3168/jds.2011-4354

Agregan, R., Echegaray, N., Lopez-Pedrouso, M., Kharabsheh. R., Franco. D., Lorenzo, J.M., 2021. Proteomic advances in milk and dairy products: a review. Molecules 26, 3832. https://doi.org/10.3390/molecules26133832

Albani, J. R., 2014a. Origin of tryptophan fluorescence lifetimes. Part 2: Fluorescence lifetimes origin of tryptophan in proteins. Journal of Fluorescence, 24, 105–117. https://doi.org/10.1007/s10895-013-1274-y

Albani, J. R., 2014b. Origin of tryptophan fluorescence lifetimes part 1. Fluorescence lifetimes origin of tryptophan free in solution. Journal of Fluorescence, 24, 93–104. http://doi.org/10.1007/s10895-013-1277-8

Ali, I., Aboul-Enein, H. Y., Singh, P., Singh, R., Sharma, B., 2010. Separation of biological proteins by liquid chromatography. Saudi Pharmaceutical Journal, 18, 59–73. https://doi.org/10.1016/j.jsps.2010.02.001

AlKhalidy, S. J., & Dosh, K. S., 2023. Isolation and purification of as-CN from sheep milk and measuring the effectiveness of its enzymatic hydrolysis in inhibiting ACE1. Chemical Methodologies, 7, 156-167. https://doi.org/10.22034/chemm.2023.366342.1618

Alonso-Fauste, I., Andrés, M., Iturralde, M., Lampreave, F., Gallart, J., Álava, M. A., 2012. Proteomic characterization by 2-DE in bovine serum and whey from healthy and mastitis affected farm animals. Journal of Proteomics, 75, 3015–3030. https://doi.org/10.1016/j.jprot.2011.11.035

Anguita, G., Martin, R., Garcia, T., Morales, P., Haza, A. I., Gonzalez, I., Sanz, B., Hernandez, P. E., 1996. Immunostick ELISA for detection of cow's milk in ewe's milk and cheese using a monoclonal antibody against bovine β-casein. Journal of Food Protection, 59, 436–437. https://doi.org/10.4315/0362-028X-59.4.436

Appuhamy, J. A. D. R. N., Bell, A. L., Nayananjalie, W. A. D., Escobar, J., Hanigan, M. D., 2011. Essential amino acids regulate both initiation and elongation of mRNA translation independent of insulin in MAC-T cells and bovine mammary tissue slices. The Journal of Nutrition, 141, 1209–1215. https://doi.org/10.3945/jn.110.136143

Basch, J. J., Douglas Jr, F. W., Procino, L. G., Holsinger, V. H., Farrell Jr, H. M., 1985. Quantitation of caseins and whey proteins of processed milks and whey protein concentrates, application of gel electrophoresis, and comparison with Harland-Ashworth procedure. Journal of Dairy Science, 68, 23–31. https://doi.org/10.3168/jds.S0022-0302(85)80792-X

Belloque, J, Smith, G. M., 1998. 1H-nuclear magnetic resonance studies on the conformational changes related to the foaming properties of β-lactoglobulin. Journal of Dairy Science, 81, 2580–2587. https://doi.org/10.3168/jds.S0022-0302(98)75815-1

Belloque, J., Ramos, M., 1999. Application of NMR spectroscopy to milk and dairy products. Trends in Food Science & Technology, 10, 313–320. https://doi.org/10.1016/S0924-2244(00)00012-1

Belloque, Josefina, & Ramos, M., 2002. Determination of the casein content in bovine milk by 31P-NMR. Journal of Dairy Research, 69, 411-418. https://doi.org/10.1017/S0022029902005630

Belton, P. S., Lyster, R. L. J., Richards, C. P., 1985. The 31 P nuclear magnetic resonance spectrum of cows' milk. Journal of Dairy Research, 52, 47–54. https://doi.org/10.1017/s0022029900023888

Bewley, M. C., Baker, E. N., Qin, B. Y., Jameson, G. B., Sawyer, L., 1997. Bovine beta-lactoglobulin and its variants: a three-dimensional structural perspective. In Milk

Protein Polymorphism Conference (Vol. II, pp. 100-109). IDF Seminar Milk Protein Polymorphism: Brussels, Belgium. https://pascalfrancis.inist.fr/vibad/index.php?action=getRecordDetail&idt=2431662

Bobe, G., Beitz, D. C., Freeman, A. E., Lindberg, G. L., 1998. Separation and quantification of bovine milk proteins by reversed-phase high-performance liquid chromatography. Journal of Agricultural and Food Chemistry, 46, 458–463. https://doi.org/10.1021/jf970499p

Bogomolov, A., Melenteva, A., 2013. Scatter-based quantitative spectroscopic analysis of milk fat and total protein in the region 400–1100nm in the presence of fat globule size variability, Chemometrics and Intelligent Laboratory Systems, 126, 129-139, https://doi.org/10.1016/j.chemolab.2013.02.006

Bonfatti, V., Grigoletto, L., Cecchinato, A., Gallo, L., Carnier, P., 2008. Validation of a new reversed-phase high-performance liquid chromatography method for separation and quantification of bovine milk protein genetic variants. Journal of Chromatography A, 1195, 101–106. https://doi.org/10.1016/j.chroma.2008.04.075

Bonfatti, V., Giantin, M., Rostellato, R., Dacasto, M., Carnier, P., 2013. Separation and quantification of water buffalo milk protein fractions and genetic variants by RP-HPLC: short communication. Food Chemistry 136, 364–367. https://doi.org/10.1016/j.foodchem.2012.09.002

Bonizzi, I., Buffoni, J. N., Feligini, M., 2009. Quantification of bovine casein fractions by direct chromatographic analysis of milk. Approaching the application to a real production context. Journal of Chromatography A, 1216, 165–168. https://doi.org/10.1016/j.chroma.2008.11.045

Bordin, G., Cordeiro Raposo, F., De La Calle, B., Rodriguez, A. R., 2001. Identification and quantification of major bovine milk proteins by liquid chromatography. Journal of Chromatography A, 928, 63–76. https://doi.org/10.1016/S0021-9673(01)01097-4

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Bramanti, E., Sortino, C., Onor, M., Beni, F., Raspi, G., 2003. Separation and determination of denatured αs1-, αs2-, β-and κ-caseins by hydrophobic interaction chromatography in cows', ewes' and goats' milk, milk mixtures and cheeses. Journal of Chromatography A, 994, 59–74. https://doi.org/10.1016/S0021-9673(03)00483-7

Chang, S. K. C., Zhang, Y., 2017. Protein analysis. In Food analysis, 315–331. https://link.springer.com/chapter/10.1007/978-3-319-45776-5_18.

Chiacchierini, E., D'Ascenzo, F., Restuccia, D., Vinci, G., 2003. Milk soluble whey proteins: fast and precise determination with Dumas method. Analytical Letters, 36, 2473–2484. https://doi.org/10.1081/AL-120024336

Church, F. C., Porter, D.H., Catignani, G, L., Swaisgood, H, E., 1985. An o-phthalaldehyde spectrophotometric assay for proteinases. Analytical biochemistry, 146, (2), 343-348. https://doi.org/10.1016/0003-2697(85)90549-4

Claverol, S., Burlet-Schiltz, O., Gairin, J. E., Monsarrat, B., 2003. Characterization of protein variants and post-translational modifications: ESI-MS analyses of intact proteins eluted from polyacrylamide gels. Molecular & Cellular Proteomics, 2, 483–493. https://doi.org/10.1074/mcp .T300003-MCP200.

Cle´ment, P., O. Agboola, S., Bencinic, R., 2006. A study of polymorphism in milk proteins from local and imported dairy sheep in Australia by capillary electrophoresis. LWT - Food Science and Technology, 39, (63-69). https://doi.org/10.1016/j.lwt.2004.11.004

Creed, D., 1984. The photophysics and photochemistry of the near-UV absorbing amino

acids–i. Tryptophan and its simple derivatives. Photochemistry and Photobiology, 39, 537–562. https://onlinelibrary.wiley.com/doi/10.1111/j.1751-1097.1984.tb03890.x

Cummins, P. M., Dowling, O., O'Connor, B. F., 2011. Ion-exchange chromatography: basic principles and application to the partial purification of soluble mammalian prolyl oligopeptidase. In Protein Chromatography, 215–228. https://doi.org/10.1007/978-1-60761-913-0_12

Cummins, P. M., Rochfort, K. D., O'Connor, B. F., 2017. Ion-exchange chromatography: basic principles and application. In Protein Chromatography, 209–223. https://doi.org/10.1007/978-1-4939-6412-3_11

Cunsolo, V., Cairone, E., Fontanini, D., Criscione, A., Muccilli, V., Saletti, R., Foti, S., 2009. Sequence determination of αs1-casein isoforms from donkey by mass spectrometric methods. Journal of Mass Spectrometry, 44, 1742–1753. https://doi.org/10.1002/jms.1683

Cunsolo, V., Muccilli, V., Saletti, R., Foti, S., 2011b. Applications of mass spectrometry techniques in the investigation of milk proteome. European Journal of Mas Spectrometry, 17, 305–320. https://doi.org/10.1255/ejms.1147

D'Alessandro, A., Zolla, L., Scaloni, A., 2011. The bovine milk proteome: cherishing, nourishing and fostering molecular complexity: an interactomics and functional overview. Molecular BioSystems, 7, 579–597. https://pubs.rsc.org/en/content/articlelanding/2011/mb/c0mb00027b

Dall'olio, S., Davoli, R., Russo, V., 1990. Affinity Chromatography of Ovine Casein. Dairy Sci. 73, 1707-1711. https://doi.org/10.3168/jds.S0022-0302(90)78847-9

Daniloski, D., McCarthy, N. A., O'Callaghan, T. F., Vasiljevic, T., 2022. Authentication of β-casein milk phenotypes using FTIR spectroscopy. International Dairy Journal, 129. https://doi.org/10.1016/j.idairyj.2022.105350

D'auria, E., Agostoni, C., Giovannini, M., Riva, E., Zetterström, R., Fortin, Greppi, G.F., Bonizzi, L., Roncada, P., 2005. Proteomic evaluation of milk from different mammalian species as a substitute for breast milk. Acta Paediatrica, 94, 1708–1713. httsp://doi.org/10.1111/j.1651-2227.2005.tb01842.x

Davies, D. T., Law, A. J. R., 1987. Quantitative fractionation of casein mixtures by fast protein liquid chromatography. Journal of Dairy Research, 54, 369–376. https://doi.org/10.1017/S0022029900025541

Davoodi, S. H., Shahbazi, R., Esmaeili, S., Sohrabvandi, S., Mortazavian, A. M., Jazayeri, S., Taslimi, A., 2016. Health-related aspects of milk proteins. Iranian Journal of Pharmaceutical Research, 15, 573–591. https://doi.org/10.22037/ijpr.2016.1897.

Day, L., Williams, R. P. W., Otter, D., Augustin, M. A., 2015. Casein polymorphism heterogeneity influences casein micelle size in milk of individual cows. Journal of Dairy Science, 98, 3633–3644. https://doi.org/10.3168/jds.2014-9285

Díaz-Carrillo, E., Muñoz-Serrano, A., Alonso-Moraga, A., Serradilla-Manrique, J. M., 1993. Near infrared calibrations for goat's milk components: Protein, total casein, αs-, β- and k-caseins, fat and lactose. Journal of Near Infrared Spectroscopy. 1, 141-146. https://doi.org/10.1255/jnirs.17

Downs, M.L., Taylor, S.L., 2010. Effects of thermal processing on the enzyme-linked immunosorbent assay (ELISA) detection of milk residues in a model food matrix. J. Agric. Food Chem. 58, 10085–10091. https://doi.org/10.1021/jf101718f

Deutsch, E. W., Lam, H., Aebersold, R., 2008. Data analysis and bioinformatics tools for tandem mass spectrometry in proteomics. Physiological Genomics, 33, 18–25. https://doi.org/10.1152/physiolgenomics.00298.2007

Dizaji, N.F., 2016. Minor whey protein purification using ion-exchange column

chromatography. PhD Thesis, University of Western Ontario, Canada. https://ir.lib.uwo.ca/etd/3685

Dufour, É., 2011. Recent advances in the analysis of dairy product quality using methods based on the interactions of light with matter. International Journal of Dairy Technology, 64, 153-165. https://doi.org/10.1111/j.1471-0307.2010.00665.x

Dupont, D., Tomé, D., 2020. Milk proteins: digestion and absorption in the gastrointestinal tract. In: Milk Proteins From Expression to Food, (3rd ed) (pp. 701-714). Elsevier Academic Press. https://hal.inrae.fr/hal-02650425/file/3- s2.0-B9780128152515000207-main.pdf.

Dupont, D., Croguennec, T., Pochet, S., 2018a. Milk proteins - analytical methods. In Reference Module in Food Science. Inc. Elsevier. https://doi.org/10.1016/b978-0-08-100596-5.22616-4

Elagamy, E. I., 2000. Effect of heat treatment on camel milk proteins with respect to antimicrobial factors: a comparison with cows' and buffalo milk proteins. Food chemistry, 68, 227-232. https://doi.org/10.1016/S0308-8146(99)00199-5

Etzion, Y., Linker, R., Cogan, U., Shmulevich, I., 2004. Determination of protein concentration in raw milk by mid-infrared fourier transform infrared/attenuated total reflectance spectroscopy. Journal of Dairy Science, 87, 2779-2788. https://doi.org/10.3168/jds.S0022-0302(04)73405-0

Fausnaugh, J. L., Regnier, F. E., 1986. Solute and mobile phase contributions to retention in hydrophobic interaction chromatography of proteins. Journal of Chromatography A, 359, 131–146. https://doi.org/10.1016/0021-9673(86)80068-1

Friedman, D.B., Hoving, S., Westermeier, R., 2009. Isoelectric focusing and two-dimentional gel electrophoresis. B.R. Richard & M.P. Deutscher (Ed.), Methods in enzymology, guide to protein purification (2nd ed) (pp. 515-540). Elsevier Inc Puplishers. https://doi.org/10.1016/S0076-6879(09)63030-5

Felfoul, I., Jardin, J., Gaucheron, F., Attia, H., Ayadi, M. A., 2017. Proteomic profiling of camel and cow milk proteins under heat treatment. Food Chemistry, 216, 161-169. https://doi.org/10.1016/j.foodchem.2016.08.007

Fessas, D., Iametti, S., Schiraldi, A., Francesco Bonomi, F., 2001. Thermal unfolding of monomeric and dimeric β-lactoglobulins. European Journal of Biochemistry, 268, 5439-5448. https://doi.org/10.1046/j.0014-2956.2001.02484.x

Flatt, P.M., 2019. Biochemistry – Defining Life at the Molecular Level. Western Oregon University, Monmouth, OR (CC BY-NC SA). https://wou.edu/chemistry/courses/online-chemistry-textbooks/ch450-and-ch451-biochemistry-defining-life-at-the-molecular-level/.

Fogliano, V., Monti, S. M., Visconti, A., Randazzo, G., Facchiano, A. M., Colonna, G., Ritieni, A., 1998. Identification of a β-lactoglobulin lactosylation site. Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology, 1388, 295–304. https://doi.org/10.1016/S0167-4838(98)00177-0

Fong, B. Y., Norris, C.S., Palmano, K.P., 2008. Fractionation of bovine whey proteins and characterisation by proteomic techniques. International Dairy Journal 18, 23–46. https://doi.org/10.1016/j.idairyj.2007.06.005

Frederiksen, P. D., Andersen, K. K., Hammershøj, M., Poulsen, H. D., Sørensen, J., Bakman, M., Qvist, K.B., Larsen, L. B., 2011. Composition and effect of blending of noncoagulating, poorly coagulating, and well-coagulating bovine milk from individual Danish Holstein cows. Journal of Dairy Science, 94, 4787–4799. https://doi.org/10.3168/jds.2011-4343

Galvani, M., Hamdan, M., Righetti, P. G., 2001. Two-dimensional gel electrophoresis/matrix-assisted laser desorption/ionisation mass spectrometry of

commercial bovine milk. Rapid Communications in Mass Spectrometry,15, 258-264. https://doi.org/10.1002/rcm.220

Geng, X.L., Tolkach, A., Otte, J., Ipsen, R., 2015. Pilot-scale purification of α- lactalbumin from enriched whey protein concentrate by anion-exchange chromatography and ultrafiltration. Dairy Sci. & Technol. 95, 353–368. https://doi.org/10.1007/s13594-015-0215-8

Ghisaidoobe, A. B. T., Chung, S. J., 2014. Intrinsic tryptophan fluorescence in the detection and analysis of proteins: a focus on förster resonance energy transfer techniques. International Journal of Molecular Sciences, 15, 22518–22538. https://doi.org/10.3390/ijms151222518

Gobbetti, M., Ferranti, P., Smacchi, E., Goffredi, F., Addeo, F., 2000. Production of angiotensin-i-converting-enzyme-inhibitory peptides in fermented milks started by Lactobacillus delbrueckii subsp. bulgaricus SS1 and Lactococcus lactis subsp. cremoris FT4. Applied and environmental microbiology, 66, 3898–3904. https://doi.org/10.1128/AEM.66.9.3898-3904.2000

Goodall, S., Grandison, A. S., Jauregi, P. J., Price, J., 2008. Selective separation of the major whey proteins using ion exchange membranes. Journal of Dairy Science, 91, 1–10. https://doi.org/10.3168/jds.2007-0539

Gomaa, M. S. H., Abdel-Aziz, M.E., Hafez, E.H., Salama, S.I., 2014. New method for determination of protein content of goat's milk and compared with different methods. J. Food and Dairy Sci, 5, 95-101. https://jfds.journals.ekb.eg/article_52745_587851febe11e7dd8554d83f5f94c832.pdf

Gonzalez-Llano, D., Polo, C., Ramos, M., 1990. Update on HPLC and FPLC analysis of nitrogen compounds in dairy products. Lait, 70, 255-277. https://hal.science/hal-00929206/document

Gryczynski, I., Wiczk, W., Johnson, M. L., Lakowicz, J. R., 1988. Lifetime distributions and anisotropy decays of indole fluorescence in cyclohexane/ethanol mixtures by frequency-domain fluorometry. Biophysical Chemistry, 32, 173–185. https://doi.org/10.1016/0301-4622(88)87005-4

Gudgin, E., Lopez-Delgado, R., Ware, W. R., 1981. The tryptophan fluorescence lifetime puzzle: a study of decay times in aqueous solution as a function of pH and buffer composition. Canadian Journal of Chemistry, 59, 1037–1044. https://doi.org/10.1139/v81-154

Hage, D. S., 1999. Affinity Chromatography: a review of clinical applications. Clinical Chemistry, 45, 593–615. https://doi.org/10.1093/clinchem/45.5.593

Hagiwara, S., Kawai, K., Anri, A., Nagahata, H., 2003. Lactoferrin concentrations in milk from normal and subclinical mastitic cows. Journal of Veterinary Medical Science, 65, 319–323. https://doi.org/10.1292/jvms.65.319

Hailu, Y., Seifu, E., Eshetu, M., Petersen, M. A., Lamestch, R., Rattary, F., Ipsen, R., 2018. Rheological and sensory properties and aroma compounds formed during ripening of soft brined cheese made from camel milk. Int. Dairy J. 81, 122–130. https://doi.org/10.1016/j.idairyj.2018.01.007

Han, X., Aslanian, A., Yates III, J. R., 2008. Mass spectrometry for proteomics. Current Opinion in Chemical Biology, 12, 483–490. https://doi.org/10.1042/BIO20200057

Haza, A.I., Morales, P., Martín, R., García, T., Anguita, G., Sanz, B., Hernández, P.E., 1999. Detection and quantification of goat's cheese in ewe's cheese using a monoclonal antibody and two ELISA formats. Journal of the science of food and agriculture, 79, 1043-1047. https://doi.org/10.1002/(SICI)1097-0010(19990515)79:7<1043::AID-JSFA326>3.0.CO;2-5

Heck, J. M. L., Olieman, C., Schenninkc, A., Valenberga, H.J.F.V., Viskerc, M.H.P.W.,

Meuldijkc, R.C.R., Hooijdonk, A.C.M.V., 2008. Estimation of variation in concentration, phosphorylation and genetic polymorphism of milk proteins using capillary zone electrophoresis. International Dairy Journal. 18, 548-555. https://doi.org/10.1016/j.idairyj.2007.11.004

Held, D., Hofe, T. (2017). Tips & Tricks GPC/SEC: Protein Analysis with Size-Exclusion Chromatography. The Column, 13, 18–23. https://www.chromatographyonline.com/view/tips-tricks-gpcsec-protein-analysis-size-exclusion-chromatography-0

Hettinga, K., Valenberg, H.V., Vries, D.S., Boeren, S., Hooijdonk, V.T., Arendonk, V.J., Vervoort, J., 2011. The host defense proteome of human and bovine milk. PloS One, 6, e19433. https://doi.org/10.1371/journal.pone.0019433

Hinz, K., O'Connor, P. M., Huppertz, T., Ross, R. P., Kelly, A. L., 2012. Comparison of the principal proteins in bovine, caprine, buffalo, equine and camel milk. Journal of Dairy Research, 79, 185–191. https:doi.org/10.1017/S0022029912000015

Hogenboom, J.A., D'Incecco, P., Fuselli, F., Pellegrino, L., 2017. Ion-exchange chromatographic method for the determination of the free amino acid composition of cheese and other dairy products: an inter-laboratory validation study. Food Anal. Methods, 10, 3137–3148. https:doi.org/10.1007/s12161-017-0876-4

Holland, J. W., Deeth, H. C., Alewood, P. F., 2004. Proteomic analysis of κ-casein micro-heterogeneity. Proteomics, 4, 743–752. https:doi.org/10.1002/pmic.200300613

Holland, J. W., Deeth, H. C., Alewood, P. F., 2005. Analysis of O-glycosylation site occupancy in bovine κ-casein glycoforms separated by two-dimensional gel electrophoresis. Proteomics, 5, 990–1002. https:doi.org/10.1002/pmic.200401098

Holland, J. W., Deeth, H. C., Alewood, P. F., 2006. Resolution and characterisation of multiple isoforms of bovine κ-casein by 2-DE following a reversible cysteine-tagging enrichment strategy. Proteomics, 6, 3087–3095. https:doi.org/10.1002/pmic.200500780

Hollar, C.M., Law, A.J.R., Dalgleish, D.G., Brown, R.J., 1991. Separation of major casein fractions using cation-exchange fast protein liquid chromatography. Journal of Dairy Sci.74, 2403-2409. https://doi.org/10.3168/jds.S0022-0302(91)78414-2

Caseins as rheomorphic proteins: interpretation of primary and secondary structures of the α S1-, β-and κ-caseins. Journal of the Chemical Society, Faraday Transactions, 89, 2683–2692. https://pubs.rsc.org/en/content/articlelanding/1993/ft/ft9938902683

Holyrod, S., 2013. The use of near infra red spectroscopy on milk and milk products. Journal of Near Infra Red Spectroscopy, 21, 311–322. https://doi.org/10.1255/jnirs.1055

Hsieh, Y-H, P., 2010. Immunoassays. In S.S. Nielsen, Food analysis, (4th ed) (pp. 301-316). New York, NY, USA: Springer Science Business Media. https://link.springer.com/chapter/10.1007/978-1-4419-1478-1_17

Hu, F., Furihata, K., Ito-Ishida, M., Kaminogawa, S., Tanokura, M., 2004. Nondestructive observation of bovine milk by NMR spectroscopy: analysis of existing states of compounds and detection of new compounds. Journal of Agricultural and Food Chemistry, 52, 4969–4974. https://doi.org/10.1021/jf049616o

Hueso, D., Fontecha, J., Gomez-Cortes, P., 2022. Comparative study of the most commonly used methods for total protein determination in milk of different species and their ultrafiltration products. Frontiers in nutrition, 9:925565. https://doi:10.3389/fnut.2022.925565

Humphrey, R. S., Jolley, K. W., 1982. 31P-NMR studies of bovine β-casein. Biochimica Et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology, 708, 294–299. https:doi.org/10.1016/0167-4838(82)90439-3

Hurley, I. P., Elyse Ireland, H., Coleman, R. C., Williams, J. H. H., 2004. Application of immunological methods for the detection of species adulteration in dairy products. International Journal of Food Science & Technology, 39, 873–878. https://doi.org/10.1111/j.1365-2621.2004.00861.x

Gapper, L.W., Copestake, D.E., Otter, D.E., Indyk, H.E., 2007. Analysis of bovine immunoglobulin G in milk, colostrum and dietary supplements: review. Analytical and Bioanalytical Chemistry, 389, 93–109. https://link.springer.com/article/10.1007/s00216-007-1391-z

Issaq, H. J., Chan, K. C., Janini, G. M., Conrads, T. P., Veenstra, T. D., 2005. Multidimensional separation of peptides for effective proteomic analysis. Journal of Chromatography B, 817, 35–47. https:doi.org/10.1016/j.jchromb.2004.07.042

Jensen, H. B., Poulsen, N. A., Andersen, K. K., Hammershøj, M., Poulsen, H. D., Larsen, L. B., 2012. Distinct composition of bovine milk from jersey and holstein-friesian cows with good, poor, or noncoagulation properties as reflected in protein genetic variants and isoforms. Journal of Dairy Science, 95, 6905–6917. https://doi.org/10.3168/jds.2012-5675

Kakalis, L. T., Kumosinski, T. F., Farrell Jr, H. M., 1990. A multinuclear, high-resolution NMR study of bovine casein micelles and submicelles. Biophysical Chemistry, 38,87-98. https://doi.org/10.1016/0301-4622(90)80043-7

Kalač, P., Samková, E., 2010. The effects of feeding various forages on fatty acid composition of bovine milk fat: a review. Czech Journal of Animal Science, 55, 521–537. https://doi.org/10.17221/2485-cjas

Kamizake, N.K.K., Gonçalves, M.M., Zaia, C.T.B.V., Zaia, D.A.M., 2003. Determination of total proteins in cow milk powder samples: a comparative study between the kjeldahl method and spectrophotometric methods. Journal of food composition and analysis, 16, 507-516. https://doi.org/10.1016/S0889-1575(03)00004-8

Kang, D, Y., Moon, J, M., Lee, S., 2011. Comparison of size-exclusion chromatography and flow field-flow fractionation for separation of whey proteins. Bull. Korean Chem. Soc, 32, 1315-1320. https:doi.org/10.5012/bkcs.2011.32.4.1315

Kasinos, M., Sabatino, P., Vanloo, B., Gevaert, K., Martins, J. C., Van der Meeren, P., 2013. Effect of phospholipid molecular structure on its interaction with whey proteins in aqueous solution. Food Hydrocolloids, 32, 312–321. https://doi.org/10.1016/j.foodhyd.2013.01.007

Kelly, S. M., Jess, T. J., Price, N. C., 2005. How to study proteins by circular dichroism. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics, 1751, 119–139. https://doi.org/10.1016/j.bbapap.2005.06.005

Kim, H.H.Y., Jimenez-Flores, R., 1994. Comparison of milk proteins using preparative isoelectric focusing followed by polyacrylamide gel electrophoresis. J Dairy Sci 77, 2177-2190. https://doi.org/10.3168/jds.S0022-0302(94)77160-5

Kim, J. I., Choi, D. Y., Row, H. K., 2003. Separation of Whey Proteins by Anion-Exchange Membranes. Korean J. Chem. Eng, 20(3), 538-541. https://link.springer.com/article/10.1007/BF02705562

Kjeldahl, J., 1883. Neue methode zur bestimmung des stickstoffs in organischen körpern. Fresenius, Zeitschrift f. anal. Chemie 22, 366–382. https://doi.org/10.1007/BF01338151

Kostanski, L. K., Keller, D. M., Hamielec, A. E., 2004. Size-exclusion chromatography: a review of calibration methodologies. Journal of Biochemical and Biophysical Methods, 58, 159—186. https://doi.org/10.1016/j.jbbm.2003.10.001

Kumar, A., Rout, P.K., Mohanty, B.P., 2013. Identification of milk protein polymorphism in indian goats by 2D gel electrophoresis. Journal of Proteomics & Bioinformatics, 6,1-4 https://www.longdom.org/open-access/identification-of-milk-protein-polymorphism-in-indian-goats-by-2d-gel-electrophoresis-32957.html

Ladenson, J. H., Purdy, W. C., 1973. Coulometric titration of proteins with silver (I). Analytica Chimica Acta, 64, 259–271. https://doi.org/10.1016/S0003-2670(01)82444-2

Lakowicz, J. R., 2013. Instrumentation for Fluorescence Spectroscopy. Lakowicz, J.R. Principles of fluorescence spectroscopy. (3rd ed) (pp. 27-6) Springer science & business media. https://doi.org/10.1007/978-0-387-46312-4_2

Li, A., Han, X., Zheng, J., Zhai, J., Cui, N., Du, P., & Xu, J., 2023. Effects of freezing raw yak milk on the fermentation performance and storage quality of yogurt. Foods, 12, 3223. https://doi.org/10.3390/foods12173223

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., 1951. Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry, 193, 265–275. https://doi.org/10.1016/S0021-9258(19)52451-6

Le, T.T., Deeth, H.C., Bhandari, B., Alewood, P.F., Holland, J.W., 2013. Quantification of lactosylation of whey proteins in stored milk powder using multiple reaction monitoring. Food Chemistry 141, 1203-1210. http://dx.doi.org/10.1016/j.foodchem.2013.03.073

Lee, J.E., Lin, T., Kang, J.W., Shin, H.Y., Lee, J.B., Jin, D.L., 2018. 2-DE and MALDI-TOF MS-based identification of bovine whey proteins in milk collected soon after parturition. Korean Journal of Agricultural Science, 45, 635-643. https://www.kci.go.kr/kciportal/ci/sereArticleSearch/ciSereArtiView.kci?sereArticleSearchBean.artiId=ART002426831

Leonil, J., Mollé, D., Fauquant, J., Maubois, J. L., Pearce, R. J., Bouhallab, S., 1997. Characterization by ionization mass spectrometry of lactosyl β-lactoglobulin conjugates formed during heat treatment of milk and whey and identification of one lactose-binding site. Journal of Dairy Science, 80, 2270–2281. https://doi.org/10.3168/jds.S0022-0302(97)76176-9

Levieux, D., Venien, A., 1994. Rapid, sensitive two-site ELISA for detection of cows' milk in goats' or ewes' milk using monoclonal antibodies. Journal of Dairy Research, 61, 91-99. https://doi.org/10.1017/S0022029900028089

Li, J., Ding, X., Chen, Y., Song, B., Zhao, S., Wang, Z., 2012. Determination of bovine lactoferrin in infant formula by capillary electrophoresis with ultraviolet detection. J. Chromatography A, 1244, 178–183. https://doi.org/10.1016/j.chroma.2012.05.004

Liao, Y., Alvarado, R., Phinney, B., Lönnerdal, B., 2011. Proteomic characterization of human milk whey proteins during a twelve-month lactation period. Journal of Proteome Research, 10, 1746–1754. https://doi.org/10.1021/pr101028k

Liu, X., Wen, F., Yang, J., Chen, L., Wei, Y., 2010. A review of current applications of mass spectrometry for neuroproteomics in epilepsy. Mass Spectrometry Reviews, 29, 197–246. https://doi.org/10.1002/mas.20243

Lönnerdal, B., 2013. Bioactive proteins in breast milk. Journal of Paediatrics and Child Health, 49, 1–7. https://doi.org/10.1111/jpc.12104

Lübke, M., Guichard, E., Tromelin, A., Le Quéré, J. L., 2002. Nuclear magnetic resonance spectroscopic study of β-lactoglobulin interactions with two flavor compounds, γ-decalactone and β-ionone. Journal of Agricultural and Food Chemistry, 50, 7094–7099. https://doi.org/10.1021/jf020513k

Lutter, P., Parisod, V., Weymuth, H., 2011. Development and validation of a method for the

quantification of milk proteins in food products based on liquid chromatography with mass spectrometric detection. Journal of AOAC International, 94, 1043–1059. https://pubmed.ncbi.nlm.nih.gov/21919337

Lu, Y., Dai, J., Zhang, S., Qiao, J., Lian, H., & Mao, L., 2023. Identification of characteristic peptides od casein in cow milk based on MALDI-TOF MS for direct adulteration detection of goat milk. 12(7), 1519. https://doi.org/10.3390/foods12071519

Madadlou, A., O'Sullivan, S., Sheehan, D., 2011. Fast protein liquid chromatography. In Protein Chromatography, 1485, 439–447. https://doi.org/10.1007/978-1-4939-6412-3_19

Maity, S., Das, D., Ambatipudi, K., 2020. Quantitative alterations in bovine milk proteome from healthy, subclinical and clinical mastitis during S. aureus infection. Journal of Proteomics, 223, 103815. https://doi.org/10.1016/j.jprot.2020.103815

Mansor, M., Al-Obaidi, J.R., Jaafar, N.N., Ismail, I.H., Zakaria, A.F., Abidin, M.A.Z., Selamat, J., Radu, S., Jambari, N.N., 2020. Optimization of protein extraction method for 2de proteomics of goat's milk. Molecules, 25,1-18. https://doi.org/10.3390/molecules25112625

Mange, A., Bellet, V., Tuaillon, E., Van de Perre, P., Solassol, J., 2008. Comprehensive proteomic analysis of the human milk proteome: contribution of protein fractionation. Journal of Chromatography B, 876, 252–256. https://doi.10.1016/j.jchromb.2008.11.003

Mao, X., Zhang, Guo-Fang., Li, C., Zhao, Yu-Cai., Wang, Ting-Ting., Duan, Chun-Ying, Wang, Jing-Ying & Liu, Li-Bo., 2017. One-step method for the isolation of α-lactalbumin and β-lactoglobulin from cow's milk while preserving their antigenicity. International journal of food properties, 20 (4), 792-800. https://doi.org/10.1080/10942912.2016.1181649

Maurmayr, A., Cecchintao, A., Grigoletto, L., Bittante, G., 2013. Detection and quantification of αs1-, αs2-, β-, κ-casein, α-lactalbumin, β-lactoglobulin and lactoferrin in bovine milk by reverse-phase high-performance liquid chromatography. Agriculturae Conspectus Scientificus, 78, 201-205. https://acs.agr.hr/acs/index.php/acs/article/view/905

Mbye, M., Mohamed, H., Raziq, A., Kamal-Eldin, A., 2021. The effects of camel chymosin and Withania coagulans extract on camel and bovine milk cheeses. Scientific Reports, 11, 13573. https://doi.org/10.1038/s41598-021-92797-6

McLafferty, F. W., Breuker, K., Jin, M., Han, X., Infusini, G., Jiang, H., Kong, X., Begley, T. P., 2007. Top-down MS, a powerful complement to the high capabilities of proteolysis proteomics. The FEBS Journal, 274, 6256–6268. https://doi.org/10.1111/j.1742-4658.2007.06147.x

Melby, J.A., Roberts, D.S., Larson, E.J., Brown, K.A., Bayne, E.F., Jin, S., Ge, Y., 2021. Novel strategies to address the challenges in top-down proteomics. J. Am. Soc. Mass Spectrom. 32, 1278−1294. https://doi.org/10.1021/jasms.1c00099

Melenteva, A., Galyanin, V., Savenkova, E., Bogomolov, A., 2016. Building global models for fat and total protein content in raw milk based on historical spectroscopic data in the visible and short-wave near infrared range. Food Chemistry, 203, 190–198. https://doi.org/10.1016/j.foodchem.2016.01.127

Meltretter, J., Wüst, J., Pischetsrieder, M., 2013. Comprehensive analysis of nonenzymatic post-translational β-lactoglobulin modifications in processed milk by ultrahigh-performance liquid chromatography–tandem mass spectrometry. Journal of Agricultural and Food Chemistry, 61, 6971–6981. https://doi.org/10.1021/jf401549j

Meltretter, J., Wüst, J., Pischetsrieder, M., 2014. Modified peptides as indicators for thermal and nonthermal reactions in processed milk. Journal of Agricultural and Food

Chemistry, 62, 10903–10915. https://doi.org/10.1021/jf503664y
Miranda, G., Mahé, M., Leroux, C., Martin, P., 2004. Proteomic tools to characterize the protein fraction of Equidae milk. Proteomics, 4, 2496–2509. https://doi.org/10.1002/pmic.200300765
Mitulović, G., Mechtler, K., 2006. HPLC techniques for proteomics analysis—a short overview of latest developments. Briefings in Functional Genomics, 5, 249–260. https://doi.org/10.1093/bfgp/ell034
Mitulović, G., 2015. New HPLC techniques for proteomics analysis: a short overview of latest developments. Journal of Liquid Chromatography & Related Technologies, 38, 390–403. https://doi.org/10.1080/10826076.2014.941266
Moeckel, U., Duerasch, A., Weiz, A., Ruck, M., Henle, T., 2016. Glycation reactions of casein micelles. Journal of Agricultural and Food Chemistry, 64, 2953–2961. https://doi.org/10.1021/acs.jafc.6b00472
Mohamed, H., Johansson, M., Lundh, A. Kamal-Eldin, A., 2020. Short communication: Casein and α-lactalbumin content of camel milk (Camelus dromedarius) determined by capillary electrophoresis. J. Dairy Sci. 103, 11094–11099. https://doi.org/10.3168/jds.2020-19122
Mohamed, H., Ranasinghe, M., Amir, N., Nagy, P., Gariballa, S., Adem, A., Kamal-Eldin, A., 2021. A study on variability of bioactive proteins in camel (Camelus dromedarius) milk: insulin, insulin-like growth factors, lactoferrin, immunoglobulin G, peptidoglycan recognition protein-1, lysozyme and lactoperoxidase. International Journal of dairy Technology, 75, 289-297. https://doi.org/10.1111/1471-0307.12836
Mohamed, H., Nagy, P., Agbaba, J. Kamal-Eldin, A., 2021. Use of near and mid-infra spectroscopy for analysis of protein, fat, lactose and total solids in raw cow and camel milk. Food Chemistry, 334, 127436. https://doi.org/10.1016/j.foodchem.2020.127436
Molina, E., Fernandez-Fournier, A., De-Frutos, M., Ramos, M., 1995. Western blotting of native and denatured bovine ~β-lactoglobulin to detect addition of bovine milk in cheese. J Dairy Sci 79, 191-197. https://doi.org/10.3168/jds.S0022-0302(96)76350-6
Morgan, F., Bouhallab, S., Mollé, D., Henry, G., Maubois, J.-L., Léonil, J., 1998. Lactolation of β-lactoglobulin monitored by electrospray ionisation mass spectrometry. International Dairy Journal, 8, 95–98. https://doi.org/10.1016/S0958-6946(98)00025-9
Myagkonosov D. S., Abramov D. V., Ovchinnikova E. G., Krayushkina V. N., 2020. Express method for assessing proteolysis in cheese and aromatic additives with cheese flavor. Food systems. 3(4): 4–10. https://doi.org/10.21323/2618-9771-2020-3-4-4-10
Nagpal, R., Behare, P., Rana, R., Kumar, A., Kumar, M., Arora, S., Morotta, F., Jain, S., Yadav, H., 2011. Bioactive peptides derived from milk proteins and their health beneficial potentials: an update. Food and Function, 2, 18–27. https://doi.org/10.1039/c0fo00016g.
Ng-Kwai-Hang, K. F., Kroeker, E. M., 1984. Rapid separation and quantification of major caseins and whey proteins of bovine milk by polyacrylamide gel electrophoresis. Journal of Dairy Science, 67, 3052–3056. https://doi.org/10.3168/jds.S0022-0302(84)81671-9
O'donnell, R., Holland, J. W., Deeth, H. C., Alewood, P., 2004. Milk proteomics. International Dairy Journal, 14, 1013–1023. https://doi.org/10.1016/j.idairyj.2004.04.004
O'Regan, J., Ennis, M. P., Mulvihill, D. M., 2009. Milk proteins. In Handbook of hydrocolloids (pp. 298–358). Elsevier Publishers. https://doi.org/10.1533/9781845695873.298

Owusu-Apenten, R.K., 2002. Food Protein Analysis - Quantitative Effects on Processing. Maecel Dekker, Inc. https://search.library.uq.edu.au/primo-explore/fulldisplay?vid=61UQ&search_scope=61UQ_All&tab=61uq_all&docid=61UQ_ALMA21103652710003131&lang=en_US&context=L

Paludetti, L. F., Jordan, K., Kelly, A. L., Gleeson, D., 2018. Evaluating the effect of storage conditions on milk microbiological quality and composition. Irish Journal of Agricultural and Food Research, 57, 52–62. https://www.jstor.org/stable/26555011

Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., Gray, T., 1995. How to measure and predict the molar absorption coefficient of a protein. Protein Science, 4, 2411–2423. https://doi.org/10.1002/pro.5560041120

Picariello, G., De Cicco, M., Nocerino, R., Paparo, L., Mamone, G., Addeo, F., Berni Canani, R., 2019. Excretion of dietary cow's milk derived peptides into breast milk. Frontiers in Nutrition, 6, 25. https://doi.org/10.3389/fnut.2019.00025

Pinho, C., Martins, Z.E., Petisca, C., Figurska, A.M., Pinho, O., Ferreira, I.M.P.L.V.O., 2012. Size exclusion and reversed-phase high-performance liquid chromatography/uv for routine control of thermal processing of cows' and donkey milk major proteins. Journal of Dairy Research, 79, 224–231. https://doi.org/10.1017/S0022029912000064

Piston, D. W., Kremers, G.-J., 2007. Fluorescent protein FRET: the good, the bad and the ugly. Trends in Biochemical Sciences, 32, 407–414. https://doi.org/10.1016/j.tibs.2007.08.003

Plank, J., Andres, P. R., Krause, I., Winter, C., 2008. Gram scale separation of casein proteins from whole casein on a source 30q anion-exchange resin column utilizing fast protein liquid chromatography (fplc). Protein Expression and Purification, 60, 176–181. https://doi.org/10.1016/j.pep.2008.04.003

Poulsen, N. A., Jensen, H. B., Larsen, L. B., 2016. Factors influencing degree of glycosylation and phosphorylation of caseins in individual cow milk samples. Journal of Dairy Science, 99, 3325–3333. https://doi.org/10.3168/jds.2015-10226

MelRamírez-Palomino, P., Fernández-Romero, J. M., Gómez-Hens, A., 2014. Rapid chromatographic determination of caseins in milk with photometric and fluorimetric detection using a hydrophobic monolithic column. Food Chemistry, 142, 249–254. https://doi.org/10.1016/j.foodchem.2013.07.046

Ribadeau-Dumas, B., Grappin, R., 1989. Milk protein analysis. Le Lait, 69, 357–416. https://doi.org/10.1051/lait:1989527

Riechel, P., Weiss, T., Ulber, R., Buchholz, H., Scheper, T., 1998. Analysis of bovine lactoferrin in whey using capillary electrophoresis (ce) and micellar electrokinetic chromatography (mekc). Advances in Experimental Medicine and Biology, 443, 33–39. https://doi.org/10.1007/978-1-4757-9068-9_4

Raynes, J.K., Vincent, D., Zawadzki, J.L., Savin, K., Mertens, D., Logan, A., Williams, R.P.W., 2018. Investigation of age gelation in UHT milk. Beverages, 4, 95. https://www.mdpi.com/2306-5710/4/4/95

Reichardt, W., & Eckert, B., 1991. To determine the protein content of milk, cheese and meat using the biuret reaction. Molecular nutrition food research, 35(7), 731-738. https://doi.org/10.1002/food.19910350716

Ren, Q.R., Zhang, H., Guo, H.Y., Jiang, L., Tian, M., Ren, F.Z., 2014. Detection of cow milk adulteration in yak milk by ELISA. J. Dairy Sci. 97, 6000–6006. https://doi.org/10.3168/jds.2014-8127

Rohm, H., Jaros, D., Rockenbauer, C., Riedler-Hellrigl, M., Uniacke-Lowe, T., Fox, P.F., 1996. Comparison of ethanol and trichloracetic acid fractionation for measurement of proteolysis in Emmental cheese. International Dairy Journal 6, 1069-1077. https://doi.org/10.1016/S0958-6946(96)00021-0

Roncada, P., Gaviraghi, A., Liberatori, S., Canas, B., Bini, L., Greppi, G. F., 2002. Identification of caseins in goat milk. Proteomics, 2, 723–726. https://doi.org/10.1002/1615-9861(200206)2:6<723::AID-PROT723>3.0.CO;2-I

Roncada, P., Piras, C., Soggiu, A., Turk, R., Urbani, A., Bonizzi, L., 2012. Farm animal milk proteomics. Journal of Proteomics, 75, 4259–4274. https://doi.org/10.1016/j.jprot.2012.05.028

Rulliere, C., Rondeau-Mouro, C., Raouche, S., Dufrechou, M., Marchesseau, S., 2013. Studies of polyphosphate composition and their interaction with dairy matrices by ion chromatography and 31P NMR spectroscopy. International Dairy Journal, 28, 102–108. https://doi.org/10.1016/j.idairyj.2012.09.005

Runthala, A., Mbye, M., Ayyash, M., Xu, Y., Kamal-Eldin, A., 2023. Caseins: versatility of their micellar organization in relation to the functional and nutritional properties of milk: a review. Molecules, 28, 5. https://doi.org/10.3390/molecules28052023

Rustandi, R. R., 2013. Hydrophobic interaction chromatography to analyze glycoproteins. Glycosylation Engineering of Biopharmaceuticals. Methods in Molecular Biology, vol. 988: (pp.211–219). Humana Press, Totowa, NJ. https://link.springer.com/protocol/10.1007/978-1-62703-327-5_13

Ryskaliyeva, A., Henry, C., Miranda, G., Faye, B., Konuspayeva, G., Martin, P., 2018. Combining different proteomic approaches to resolve complexity of the milk protein fraction of dromedary, bactrian camels and hybrids, from different regions of Kazakhstan. PLoS ONE, 13, 1–26. https://doi.org/10.1371/journal.pone.0197026.

Rysova, L., Cejnar, P., Hanus, O., Legarova, V., Havlik, J., Nejeschlebova, H., Nemeckova, I., Jedelska, R., & Bozik, M., 2022. Use of MALDI-TOF MS technology to evaluate adulteration of small ruminant milk with raw bovine milk. Journal of dairy science, 105 (6), 4882-4894. https://doi.org/10.3168/jds.2021-21396

Sakamoto, S., Putalun, W., Vimolmangkang, S., Phoolcharoen, W., Shoyama, Y., Tanaka, H., Morimoto, S., 2018. Enzyme-linked immunosorbent assay for the quantitative/qualitative analysis of plant secondary metabolites. Journal of Natural Medicines, 72, 32–42. https://link.springer.com/article/10.1007/s11418-017-1144-z

Saliha, S.A.Z., Dalila, A., Chahra, S., Saliha, B.H., Abderrahmane, M., 2013. Separation and characterization of major milk proteins from algerian dromedary (Camelus dromedarius). Emir. J. Food Agric. 25, 283-290. https://doi.org/10.9755/ejfa.v25i4.15496

Sargent, M. G., 1987. Fiftyfold amplification of the lowry protein assay. Analytical Biochemistry, 163, 476–481. https://doi.org/10.1016/0003-2697(87)90251-X

Santos, M. J., Teixeira, J. A., Rodrigues, L. R., 2012. Fractionation of the major whey proteins and isolation of β-lactoglobulin variants by anion exchange chromatography. Separation and Purification Technology, 90, 133–139. https://doi.org/10.1016/j.seppur.2012.02.030

Sawyer, L., Barlow, P. N., Boland, M. J., Creamer, L. K., Denton, H., Edwards, P. J. B., Holt, C., Jameson, G.B., Kontopidis, G., Norris, G.E., Uhrinova, S., Wu, Su-Ying., 2002. Milk protein structure—what can it tell the dairy industry? International Dairy Journal, 12, 299–310. https://doi.org/10.1016/S0958-6946(02)00025-0

Seibert, B., Erhardt, G., Senft, B., 1985. Procedure for simultaneous phenotyping of genetic variants in cow's milk by using isoelectric focusing. Animal blood groups biochemical genetics. 16, 183-191. https://doi.org/10.1111/j.1365-2052.1985.tb01469.x

Sharma, G., Rout, D. P., Singh, D. G., 2017. Characterization of goat milk protein and comparison of milk proteins using polyacrylamide gel electrophoresis. International Journal of Scientific Engineering and Applied Science, 4, 13-18.

https://ijseas.com/volume3/v3i5/ijseas20170502.pdf
Silvestre, M. P. C., 1997. Review of methods for the analysis of protein hydrolysates. Food Chemistry, 60, 263–271. https://doi.org/10.1016/S0308-8146(96)00347-0
Simonne, A.H., Simonne, E.H., Eitenmiller, R.R., Mills, H.A., Cresman III, C.P., 1999. Could the dumas method replace the kjeldahl digestion for nitrogen and crude protein determinations in foods? J Sci Food Agric, 73, 39-45 https://doi.org/10.1002/(SICI)1097-0010(199701)73:1<39::AID-JSFA717>3.0.CO;2-4
Siuti, N., & Kelleher, N. L., 2007. Decoding protein modifications using top-down mass spectrometry. Nature Methods, 4, 817–821. https://www.nature.com/articles/nmeth1097
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K, Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., Klenk, D. C., 1985. Measurement of protein using bicinchoninic acid. Anal Biochem. 150, 76-85. https://doi.org/10.1016/0003-2697(85)90442-7
Sørensen, L.K., Lund, M., Juul, B., 2003. Accuracy of fourier transform infrared spectrometry in determination of casein in dairy cows' milk. Journal of Dairy Research, 70, 445 - 452. https://doi.org/10.1017/s0022029903006435
Soyeurt, H., Grelet, C., McParland, S., Calmels, M.V., Coffey, M., Tedde, A., Delhez, P., Dehareng, F., Gengler, N., 2020. A comparison of 4 different machine learning algorithms to predict lactoferrin content in bovine milk from mid-infrared spectra. Journal of dairy science, 103, 11585-11596. https://doi.org/10.3168/jds.2020-18870
Špoljarić, J., Mikulec, N., Plavljanić, D., Radeljević, B., Havranek, J., Antunac, N., 2013. Proving the adulteration of ewe and goat cheeses with cow milk using the reference method of isoelectric focusing of γ-casein. Mljekarstvo 63, 115-121. https://hrcak.srce.hr/106581
Starovojtova, K., Tereshchuk, L., Tarlyun, M., Dolgolyuk, I., 2020. Development of an integrated technology for milk processing with the production of functional dairy products and biotech products for food and pharmaceutical industry. International Scientific Conference. (Vol. V, pp.390–395). Prospects for the Development and Modernization of the Russian Agro-Industrial Sector (TFTS 2019). https://doi.org/10.2991/assehr.k.200113.208
Stanley, J., 2002. Precipitation: single radial immunodiffusion: laboratory technique 12-1: radial immunodiffusion test", Essentials of immunology & serology. (pp.172–174). Albany, New York. https://archive.org/details/essentialsofimmu0000stan/mode/1up
Steen, H., Mann, M., 2004. The ABC's (and XYZ's) of peptide sequencing. Nature Reviews Molecular Cell Biology, 5, 699–711. https://www.nature.com/articles/nrm1468
Strickland, M., Johnson, M.E., Broadbent, J.R., 2001. Qualitative and quantitative analysis of proteins and peptides in milk products by capillary electrophoresis. Electrophoresis, 22, 1510–1517. https://pubmed.ncbi.nlm.nih.gov/11386663
Sturaro, A., De Marchi, M., Masi, A., Cassandro, M., 2016. Quantification of whey proteins by reversed phase-HPLC and effectiveness of mid-infrared spectroscopy for their rapid prediction in sweet whey. Journal of Dairy Science, 99, 68–76. https://doi.org/10.3168/jds.2014-9077
Swaminathan, R., Krishnamoorthy, G., Periasamy, N., 1994. Similarity of fluorescence lifetime distributions for single tryptophan proteins in the random coil state. Biophysical Journal, 67(5), 2013–2023. https://doi.org/10.1016/S0006-3495(94)80685-X
Tanaka, N., Kunugi, S., 1996. Effect of pressure on the deuterium exchange reaction of α-

lactalbumin and β-lactoglobulin. International Journal of Biological Macromolecules, 18, 33–39. https://doi.org/10.1016/0141-8130(95)01053-X

Tang, J., Gao, M., Deng, C., Zhang, X., 2008. Recent development of multi-dimensional chromatography strategies in proteome research. Journal of Chromatography B, 866, 123–132. https://doi.org/10.1016/j.jchromb.2008.01.029

Taylor, J. K., Smith, S.W., 1959. Precise coulometric titration of acids and bases. Journal of research of the national bureau of satndards - A. Physics and chemistry, 63A, 153-159. https://doi.org/10.6028/jres.063A.008

Teale, F. W., Weber, G., 1957. Ultraviolet fluorescence of the aromatic amino acids. The Biochemical Journal, 65, 476–482. https://doi.org/10.1042/bj0650476

Tidona, F., Criscione, A., Guastella, A. M., Zuccaro, A., Bordonaro, S., Marletta, D., 2009. Bioactive peptides in dairy products. Italian Journal of Animal Science, 8, 315–340. https://doi.org/10.4081/ijas.2009.315.

Timasheff, S. N., Townend, R., 1964. Structure of the β-lactoglobulin tetramer. Nature, 203, 517–519. https://www.nature.com/articles/203517a0

Thompson, A., Boland, M., Singh, H., 2009. Milk proteins: from expression to food. Amsterdam, Boston, USA: Academic Press. https://ubblab.weebly.com/uploads/4/7/4/6/47469791/milk_proteins_from_expression_to_food.pdf

Trieu-Cuot P., Gripon J.C., 1981. Electro focusing and two-dimensional electrophoresis of bovine caseins. J. Dairy Res. 48, 303-310. https://doi.org/10.1017/S0022029900021725

Trimboli, F., Costanzo, N., Lopreiato, V., Ceniti, C., Morittu, V.M., Spina, A., Britti, D., 2019. Detection of buffalo milk adulteration with cow milk by capillary electrophoresis analysis. J. Dairy Sci. 102, 5962–5970. https://doi.org/10.3168/jds.2018-16194

Vincent, D., Ezernieks, V., Elkins, A., Nguyen, N., Moate, P.J., Benjamin G. Cocks., B.G., Richford, S., 2015. Milk bottom-up proteomics: method optimization. Frontiers in Genetics, 6, 1-24. https://doi.org/10.3389/fgene.2015.00360

Vincent, D., Elkins, A., Condina, M.R., Ezernieks,V., Rochfort, S., 2016. Quantitation and identification of intact major milk proteins for high-throughput lc-esi-q-tof ms analyses. Plos one, 11, 1-21. https://doi.org/10.1371/journal.pone.0163471

Vincent, D., Mertens, D., Rochfort, S., 2018. Optimization of milk protein top-down sequencing using in-source collision-induced dissociation in the maxis quadrupole time-of-flight mass spectrometer. Molecules. 23, 1-22. https://doi.org/10.3390/molecules23112777

Vera-Bravo, R., Hernández, A.V., Peña, S., Alarcón, C., Loaiza, A.E., Celis, C.A., 2022. Cheese whey milk adulteration determination using casein glycomacropeptide as an indicator by HPLC. Foods, 11, 3201. https://doi.org/10.3390/foods11203201

Voswinkel, L., Kulozik, U., 2011. Fractionation of whey proteins by means of membrane adsorption chromatography. Procedia Food Science, 1, 900–907. https://doi.org/10.1016/j.profoo.2011.09.136

Wahlgren, N. M., Drakenberg, T., 1995. Milk. In Annual reports on NMR spectroscopy, 31, 275–312. https://doi.org/10.1016/S0066-4103(08)60152-0

Warren, C. M., Jhaveri, S., Warrier, M. R., Smith, B., Gupta, R. S., 2013. The epidemiology of milk allergy in US children. Annals of Allergy, Asthma and Immunology, 110, 370–374. https://doi.org/10.1016/j.anai.2013.02.016.

Weinbrenner, W. F., Etzel, M. R., 1994. Competitive adsorption of α-lactalbumin and bovine serum albumin to a sulfopropyl ion-exchange membrane. Journal of Chromatography A, 662, 414–419. https://doi.org/10.1016/0021-9673(94)80530-X

Wu, D., He, Y., Feng, S., & Sun, D.-W., 2008. Study on infrared spectroscopy technique for last measurement of protein content in milk powder based on LS-SVM. Journal of food engineering, 84, 124-131. https://doi.org/10.1016/j.jfoodeng.2007.04.031

Wu, H.-Y., Tseng, V. S.-M., Liao, P.-C., 2007. Mining phosphopeptide signals in liquid chromatography-mass spectrometry data for protein phosphorylation analysis. Journal of Proteome Research, 6, 1812–1821. https://doi.org/10.1021/pr060631d

Yakubu, H. G., Kovacs, Z., Toth, T., Bazar, G., 2022. The recent advances of near-infrared spectroscopy in dairy production: a review. Critical Reviews in Food Science and Nutrition, 62, 810-831. https://doi.org/10.1080/10408398.2020.1829540

Yamada, M., Murakami, K., Wallingford, J. C., Yuki, Y., 2002. Identification of low-abundance proteins of bovine colostral and mature milk using two-dimensional electrophoresis followed by micro-sequencing and mass spectrometry. Electrophoresis, 23, 1153–1160. https://pubmed.ncbi.nlm.gov/11981865/

Yang, Y., Bu, D., Zhao, X., Sun, P., Wang, J., Zhou, L., 2013. Proteomic analysis of cow, yak, buffalo, goat, and camel milk whey proteins: quantitative differential expression patterns. Journal of Proteome Research, 12, 1660–1667. https://doi.org/10.1021/pr301001m

Yasmin, I., Iqbal, R., Liaqat, A., Khan, W.A., Nadeem, M., Iqbal,A., Chughtai, M.F.J., Rehman, S.J.U., Tehseen, S., Mehmood, T., Ahsan, S., Tanweer,S., Naz, S., Khaliq, A., 2020. Characterization and comparative evaluation of milk protein variants from pakistani dairy breeds. Food Sci Anim Resour. 40, 689–698. https://doi.org/10.5851/kosfa.2020.e44

Ye, X., Yoshida, S., Ng, T. B., 2000. Isolation of lactoperoxidase, lactoferrin, α-lactalbumin, β-lactoglobulin B and β-lactoglobulin A from bovine rennet whey using ion exchange chromatography. The International Journal of Biochemistry & Cell Biology, 32, 1143–1150. https://doi.org/10.1016/s1357-2725(00)00063-7

Yelubaeva, M. Y., Buralkhiev, B. A., Serikbayeva, A. D., Narmuratova, M. H., Kenenbay, S. Y., 2017. Electrophoretic identification of casein in various types of milk. Journal of Biological Sciences, 17, 348-352. https://doi.org/10.3844/ojbsci.2017.348.352

Yoshida, S., 1990. Isolation of β-lactoglobulin and α-lactalbumin by gel filtration using sephacryl S-200 and purification by diethylaminoethyl ion-exchange chromatography. Journal of Dairy Science, 73, 2292-2298. https://doi.org/10.3168/jds.S0022-0302(90)78910-2

Yüksel, Z., Erdem, Y. K., 2010. Detection of the milk proteins by RP-HPLC. GIDA-Journal of Food, 35, 5–11. https://www.academia.edu/10054621/DETECTION_OF_THE_MILK_PROTEINS_BY_RP-HPLC

Zagorchev, L., Dimitrova, M., Odjakova, M., Teofanova, D., Hristov, P., 2013. Electrophoretic characterization of milk proteins from bulgarian rhodopean cattle. Bulgarian Journal of Agricultural Science, 19, 197–200. https://agrojournal.org/19/02-50s.pdf

Zappacosta, F., Di luccia, A., Ledda, L., Addeo, F., 1998. Identification of C-terminally truncated forms of β-lactoglobulin in whey from romagnola cows' milk by two dimensional electrophoresis coupled to mass spectrometry. Journal of Dairy Research, 65, 243–252. https://doi.org/10.1017/s0022029997002859

Zhang, Y., Luo, J., Bi, J., Wang, J., Sun, L., Liu, Y., Zhang, G., Ma, G., Su, Z., 2010. Efficient separation of homologous α-lactalbumin from transgenic bovine milk using optimized hydrophobic interaction chromatography. Journal of Chromatography A,

1217, 3668–3673. https://doi.org/10.1016/j.chroma.2010.03.060
Zhao, P., Zare, R. N., Chen, H., 2019. Absolute quantitation of oxidizable peptides by coulometric mass spectrometry. Journal of The American Society for Mass Spectrometry, 30, 2398–2407. https://doi.org/10.1007/s13361-019-02299-z
Zhou, H., Xu, S., Ye, M., Feng, S., Pan, C., Jiang, X., Li, X., Han, G., Fu, Y., Zou, H., 2006. Zirconium phosphonate-modified porous silicon for highly specific capture of phosphopeptides and MALDI-TOF MS analysis. Journal of Proteome Research, 5, 2431–2437. https://doi.org/10.1021/pr060162f

Table 1: Summary of total milk/dairy products protein quantitation methods

Assay	**Principle**	**Assay sensitivity (Concentration range of BSA)**	**Advantages**	**Limitations**	**References**
UV Absorbance	Total milk/dairy proteins based on tryptophan, tyrosine, phenylalanine, and S-S residues absorption at 280 nm.	50 -2000 µg/ml	Simple method. Sample can be measured directly.	Protein responses are dependent on amino acid composition.	Kuaye (1994)
Biuret	Polypeptide chains in protein react with copper (II) ion in the presence of sodium hydroxide turning the solution purple 540 nm.	150-900 µg/ml	Simple method.	Low sensitivity. This assay may be interfered with amino acid, tris-amino methane, and ammonium ions.	Reichardt & Eckert (1991)
Bicinchoninic Acid (BCA)	Colorimetric assay for total milk/dairy proteins based on the formation of complexes between Cu	20-2000 µg/µl	Simple method. High sensitivity.	This assay may be interfered with ammonium sulfate, thiol, and phospholipid.	Hueso et al. (2022)

Assay	Principle	Assay sensitivity (Concentration range of BSA)	Advantages	Limitations	References
	ions and cysteine, tryptophan, tyrosine, and phenylalanine. The absorbance is enhanced by BCA with the addition of higher sensitivity and selectivity. Absorbance is measured at 560 nm.				
Bradford	Colorimetric assay for total milk/dairy proteins based on the binding of tryptophan, tyrosine, and phenylalanine to the Coomassie Brilliant Blue G250. Absorbance is measure at 595-600 nm.	10-2000 µg/µl	Simple method.	Protein responses are dependent on their amino acid composition. Surfactant can interfere with the chromogenic reaction through contamination.	Hueso et al. (2022)
Lowry	This assay is for total milk/dairy proteins. Molybdenum & phosphor-tungstic acids of a phenol reagent are reduced by tryptophan, cysteine and tyrosine residues,	5-200 µg/µl	Sensitive method	The determination can be interfered-with due to the contamination of reducing agents. Protein responses are dependent on their amino	Kamizake et al. (2003)

Assay	Principle	Assay sensitivity (Concentration range of BSA)	Advantages	Limitations	References
	turning alkaline copper solution blue (750 nm)			acid composition.	

*BSA: Bovine Serum Albumin. Modified from: https://jascoinc.com/applications/protein-quantitation-spectrophotometer

Table 2: Selected reversed-phase HPLC methods for the separation of milk proteins

Column	Column parameters	Mobile phase	Detection	Analysis	Reference
Vydac C4 (Dionex)	150×2.1 mm, particle size 5 µm, pore diameter 300 Å, 40°C	Solvent A- acetonitrile: water: trifluoroacetic acid 100:899: 1 (v/v/v) Solvent B- acetonitrile: water: trifluoroacetic acid 899:100: 1 (v/v/v) A programed gradient starting from 26.5% B to 43.3% B in 56 min, at a flow rate of 0.25 ml/ min	DAD (240 nm)	Proteins in skimmed bovine milk powder (α-, β-, and κ-caseins, α-lactalbumin, and β-/actoglobulin A, and B)	Bordin et al. (2001)
Jupiter C4 (Phenomenex)	250 mm × 2 mm, particle size 5µm, pore diameter 300 Å, 35°C	Solvent A- water : trifluoroacetic acid 999:1 (v/v) Solvent B- acetonitrile : trifluoroacetic acid 999:1 (v/v) Programmed gradient starting from 31-45% B at 0.3% B/min.	DAD (214 nm) MS- (ion mode (polarity):	Proteins in skimmed bovine milk (αs1-, αs2-, β-, and κ-caseins, β-lactoglobulin, and α-lactalbumin)	Frederiksen et al. (2011), Jensen et al. (2012), Poulsen, Jensen & Larsen (2016)
Jupiter C4 (Phenomenex)	250mm×4.6mm, particle size 5µm, pore diameter 300 Å, Room Temperature	Solvent A- water : trifluoroacetic acid 999:1 (v/v) Solvent B- acetonitrile : trifluoroacetic acid 999:1 (v/v) Programed gradient starting from 0-100% B in 46 min	UV detection- 220 nm	Proteins in skimmed bovine milk (αs1-, αs2-, β-, and κ-caseins)	Bonizzi, Buffoni, & Feligini, (2009)
Discovery Wide Pore C5 (Supelco)	150 x 2.1 mm, particle size 3µm, pore diameter 300 Å, 52°C	Solvent A- water : trifluoroacetic acid 997.5:2.5 (v/v) Solvent B- acetonitrile : trifluoroacetic acid 998:2 (v/v)	UV detection at 214 nm	Camel milk proteins	Ryskaliyeva et al. (2018)

		A programmed gradient from 5-41% B in 36 min at a flow rate of 0.2 mL/min.			
Zorbax 300SB C8 (Agilent Technologies)	150 x 4.6 mm, particle size 3.5µm, pore diameter 300 Å, 45°C	Solvent A- water : trifluoroacetic acid 999:1 (v/v) Solvent B- acetonitrile : trifluoroacetic acid 999:1 (v/v) A programmed gradient from 33-45% B in 36 min at a flow rate of 0.5 ml/min	UV detection at 214 nm	Bovine milk caseins and whey proteins	Bonfatti et al. (2008)
Zorbax 300SB C8 (Agilent Technologies)	75 x 2.1 mm, particle size 5µm, pore diameter 300 Å, 70°C	Solvent A- acetonitrile, water: trifluoroacetic acid 50:949:1 (v/v/v) Solvent B- acetonitrile : trifluoroacetic acid 999:1 (v/v). Programmed gradient starting from 5-50% B in 9 min at a flow rate of 2.5 ml/min	Diode array & UV at 214 nm	Sweet whey from pasteurized milk	Sturaro et al. (2016)
Aeris Widepore XB-C8, (Phenomenex)	250 x 2.1 mm, particle size 3/6µm, pore diameter 300 Å, 70°C	Solvent A- acetonitrile: water: trifluoroacetic acid 50:949: 1 (v/v/v) Solvent B- acetonitrile: trifluoroacetic acid 999:1 (v/v) Programmed gradient from 20-45 % B in 18 min at a flow rate was 0.5 ml/min	Diode Array Detector UV at 214 nm	Bovine milk proteins (αs1- , αs2-, β-, and κ-caseins, α-lactalbumin, β-lactoglobulin, and lactoferrin)	Maurmayr et al. (2013)
Hi-Pore C18 (BioRad)	(250 x 4.6 nm) particle size 5 µm, pores 300 Å	Solvent A-0.1% formic acid in water (v/v)	UV detection at 280 nm	Caseins of bovine skim milk	Day et al. (2015)

		Solvent B-0.1% formic acid in acetonitrile (v/v) Programmed gradient from 25-50% in 40 minutes			
Onyx monolithic C18 (Phenomenex)	(100 x 4.6 mm) particle size 13 nm & 2 µm	Solvent A-water: trifluoroacetic acid 999:1 (v/v) Solvent B-acetonitrile: water: trifluoroacetic acid 950:49: 1 (v/v/v) Linear gradient starting 2.5-10 B at a flow rate of 3 ml/min	Diode array (220nm) and fluorescence (Ex. 280nm, Em. 340nm)	Whole, skimmed and fortified bovine milk caseins	Ramírez-Palomino et al. (2014)
Zorbax 300SB C18 (Agilent Technologies)	250 x 4.6 mm, particle size 5 µm, pore size 30 nm, 25°C	Solvents A: acetonitrile : water: trifluoroacetic acid 100:900:1 (v/v/v) Solvent B: Acetonitrile : water : trifluoroacetic acid 900:100:1 (v/v/v) Linear gradient, total run time; 30 minutes. Flow rate; 1.0 ml/min.	UV-detector 220 nm	Raw and pasteurized bovine milk proteins (α-, β-, and κ-, caseins, α-lactalbumin, and β-Lactoglobulin)	Yüksel & Erdem (2009)
Microsorb-MV C18 (Agilent Technologies)	250 x 4.6 mm, particle size 5 µm, pore size 30 nm, Room Temperature	Solvents A: acetonitrile : water : trifluoroacetic acid 100:900:1 (v/v/v) Solvent B: acetonitrile : water : trifluoroacetic acid 900:100:1 (v/v/v) Programmed gradient starting from 27-45% B	UV-detector 220 nm	Bovine milk proteins (αs1-, αs2-, β-, and κ-caseins, α-lactalbumin, and β-lactoglobulin)	Bobe et al. (1998)

		in 43 min at a flow rate: 1.2 ml/min.			
Poroshell 300SB C18 (Agilent Technologies)	2.1 × 75 mm, particle size 5 µm, pore size 30 nm, 25°C	Solvent A- water : trifluoroacetic acid 999:1 (v/v) Solvent B- acetonitrile : trifluoroacetic acid 999:1 (v/v) Programmed gradient from 0-50% B over 55 min at a flow rate of 0.50 mL/min.	DAD (214 nm)	Caseins in raw bovine milk	Paludetti et al.(2018)

Table 3: Protein identification techniques, and their application in milk and dairy product protein analysis

Technique	Applications, Advantages, Limitations	References
Kjeldahl method	Crude total protein content in milk through nitrogen determination and use of correction factor (6.25 or 6.38), therefore may not be accurate. Not commonly used nowadays as it uses harsh chemicals and requires long times. This method cannot differentiate between protein and non-protein nitrogen.	Gomma, (2014)
Spectrophotometric methods	Total protein content as BSA equivalents. Simple and fast methods	See Table (1)
Reversed Phase Liquid Chromatography	Separation and quantification of individual proteins based on polarity. Requires standard reference proteins for identification and calibration. Useful for authentication, detection of adulteration, and effect of processing on milk proteins.	Ryskaliyeva et al. (2018), Reynes et al. (2018), Vera-Bravo et al. (2022)
Hydrophobic Interaction Chromatography	Separation and quantification of individual proteins based on hydrophobicity. Requires standard reference proteins for identification and calibration. Useful for authentication, detection of adulteration, and effect of processing on milk proteins.	Bramanti et al. (2003) Santos et al. (2011)
Ion Exchange Chromatography	Mainly used for the separation and purification of milk proteins based on protein anionic or cationic charges. Requires standard reference proteins for identification. Useful for preparative chromatography.	Santos et al. (2012), AlKhalidy & Dosh (2023)
Size Exclusion Chromatography	Mainly used for the separation and purification of milk proteins based on proteins' molecular size. Requires standard reference proteins for identification. Useful for preparative chromatography.	Pinho, (2012)
Affinity chromatography	Highly selective method used for the separation and purification of milk proteins based on special affinity or interaction. Mainly used for preparation of enzymes and low abundant proteins.	Dall'olio, (1990)
Gel electrophoresis (One- and two-dimensional)	Mainly used for the separation of proteins based on electrical charge, which is mainly determined by size. Requires standard marker proteins for determination of molecular weight. Provides characteristic protein profiles that can be used to illustrate differences between sources and effects of processing.	Yelubaeva et al. (2017), Sharma et al. (2017), Yasmin et al. (2020), Mbye et al. (2021)
Capillary electrophoresis	Separation and quantification of individual milk proteins based on electrical charge/molecular size. Requires standard reference proteins for identification and calibration. Useful for authentication, detection of adulteration, and effect of processing on milk proteins.	Trimboli et al. (2019), Mohamed et al. (2020)
Western blotting	Uses specific antibodies to detect selected proteins, e.g. immunologically-active & allergic protein.	Picariello et al. (2019), Maity, Das & Ambatipudi, (2020)
Infra-Red Spectroscopy	Used for the quantification of total or individual milk proteins after multivariate calibration (Chemometrics	Mohamed et al. (2020),

	models). Useful for authentication, detection of adulteration, and effect of processing on milk proteins	Soyeurt et al. (2020), Yakubu, (2022), Daniloski et al. (2022)
Enzyme-Linked Immuno-Sorbent Assays (ELISA)	Selective methods for the quantification of individual proteins based on body-antibody direct or indirect binding. Useful for the detection of species-specific protein epitopes.	Mohamed et al. (2021)
Nuclear Magnetic Resonance Spectrometry (NMR)	Allows detecting structural changes in proteins or protein fractions on basis of functional groups. Useful in detecting the effect of processing in milk proteins.	Rulliere et al. (2013)
Mass spectrometry (MS)	A very important technique for protein identification and quantification based on mass fragmentation patters. Required for the identification of unknown proteins and peptides and can be used in bottom-up and top-down approaches. Coupled with bioinformatics, it is used for the obtention of protein sequences, protein identification, and post-translation modification sites.	Vincent et al. (2015), Vincent et al. (2016), Vincent et al. (2018), Raynes et al. (2018), Rysova et al. (2022), Lu et al. (2023)

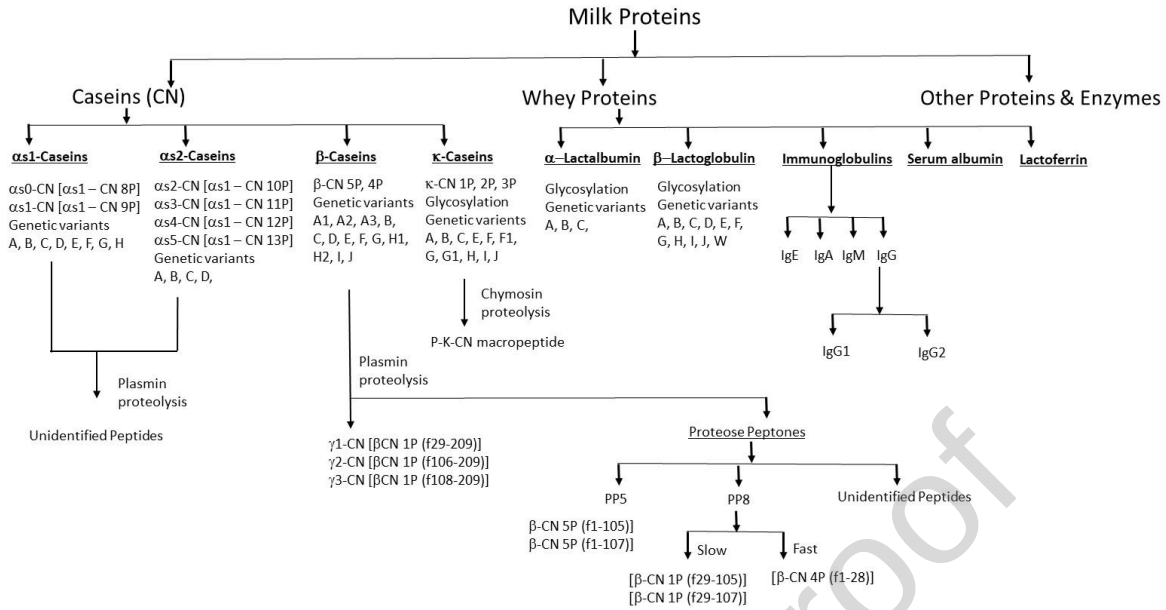


Figure 1: Classification of the major milk proteins (adapted from O'Regan et al., 2009 with permission from Elsevier)

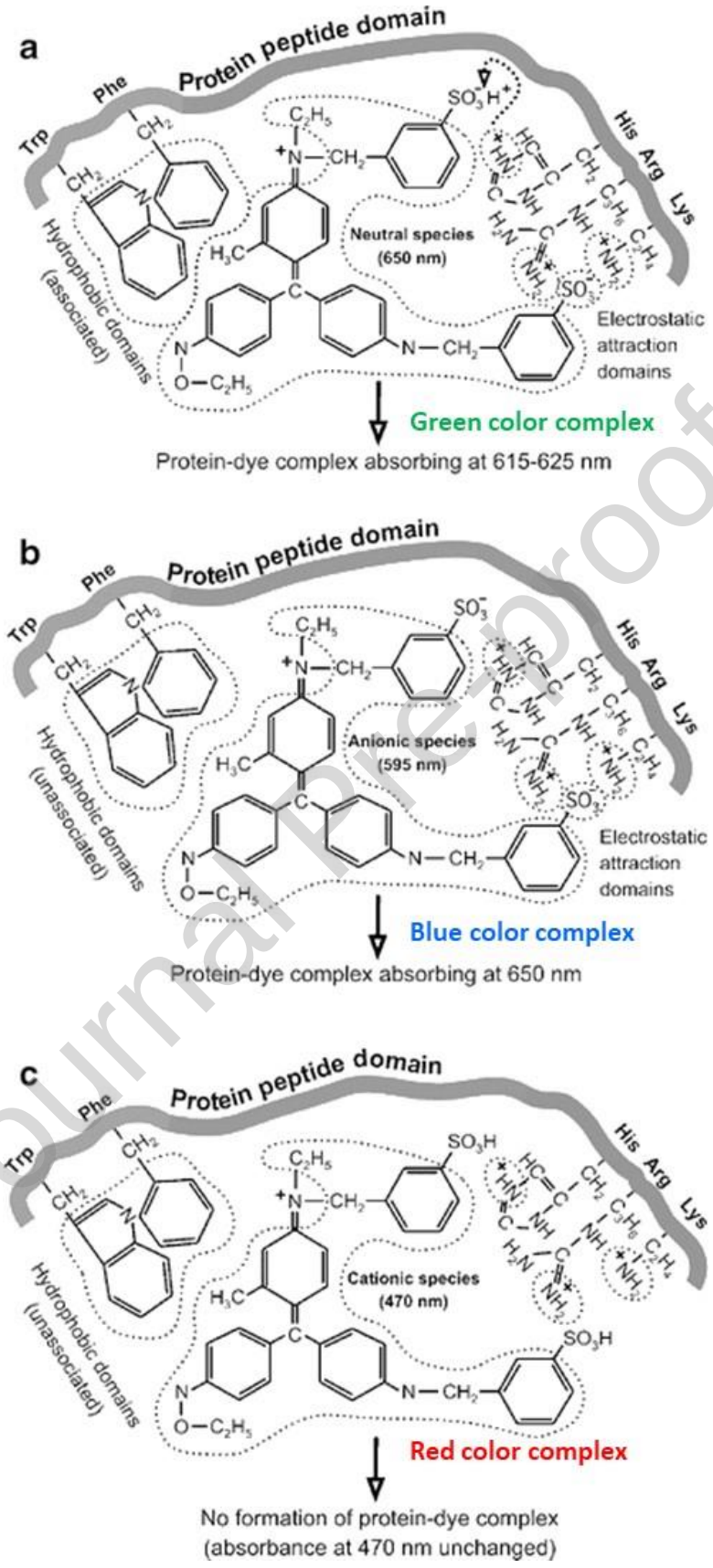


Figure 2: Mechanism of Coomassie brilliant blue dye-binding with proteins (a) neutral residues, (b) anionic residues, and (c) cationic residues. Reproduced from Georgiou et al. (2008) with permission from Elsevier with modification.

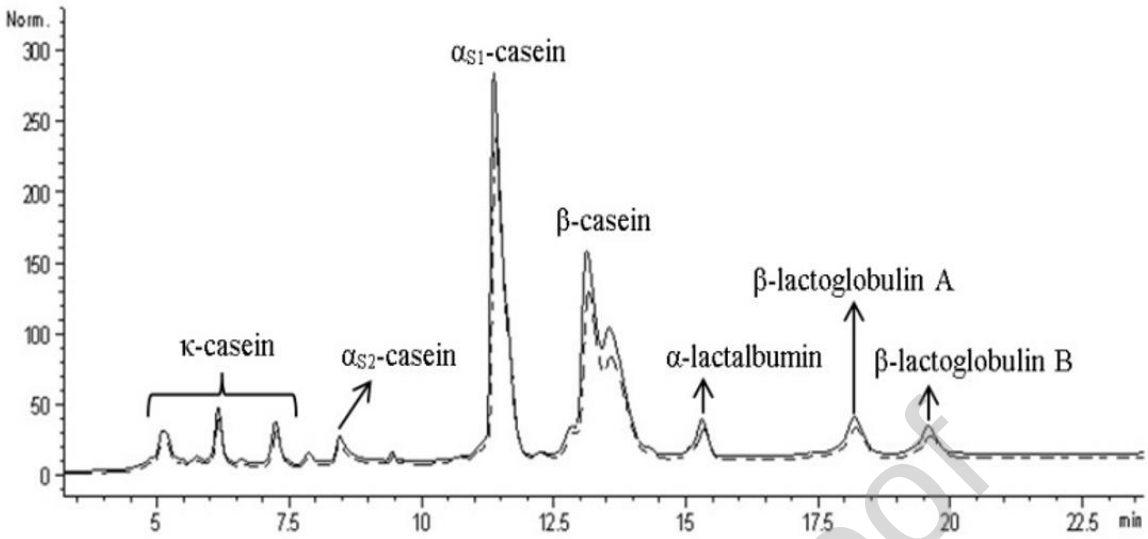
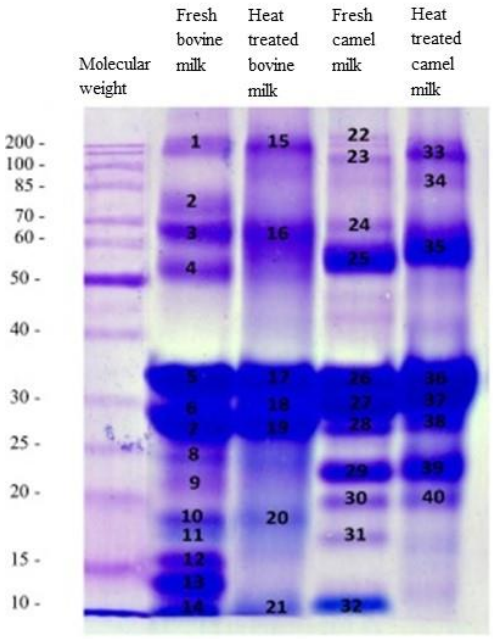


Figure 3: RP-HPLC separation of bovine milk protein by Poroshell 300SB C18 column. Reproduced from Paludetti et al. (2018) with creative commons permission.



Band	Identified protein*	Band	Identified protein*
1	Complement C3	21	α_{S1}-CN
2	LTF	22	XDO
3	PIgR	23	Uncharacterized protein
4	BSA	24	LF
5	α_{S1}-CN	25	CSA
6	κ-CN	26	α_{S1}-CN
7	κ-CN	27	β-CN
8	α_{S1}-CN	28	α_{S2}-CN
9	Uncharacterized protein	29	GLYCAM1
10	α_{S2}-CN	30	GLYCAM1
11	α_{S1}-CN	31	PGRP
12	β-lg	32	α-la
13	β-lg	33	α_{S1}-CN
14	α-la	34	α_{S2}-CN
15	α_{S1}-CN	35	α_{S1}-CN
16	α_{S2}-CN	36	α_{S1}-CN
17	α_{S1}-CN	37	β-CN
18	α_{S1}-CN	38	α_{S2}-CN
19	κ-CN	39	GDCAM
20	α_{S2}-CN	40	α_{S1}-CN

*Identified protein from the band based with the best e-value.
LTF : Lactotransferrin ; PIgR : Polymeric immunoglobulin receptor ; BSA : Bovine serum albumin ; XDO : Xanthine dehydrogenase/oxidase ; LF : Lactoferrin ; CSA : Camel serum albumin ; GLYCAM1 : Glycosylation-dependent cell adhesion molecule 1 ; PGRP :Peptidoglycan recognition protein 1.

Figure 4: Electrophoretic separation of fresh and heated (80°C/60 min) bovine and camel milks by SDS-PAGE. Reproduced from Felfoul et al. (2017) with creative commons permission.

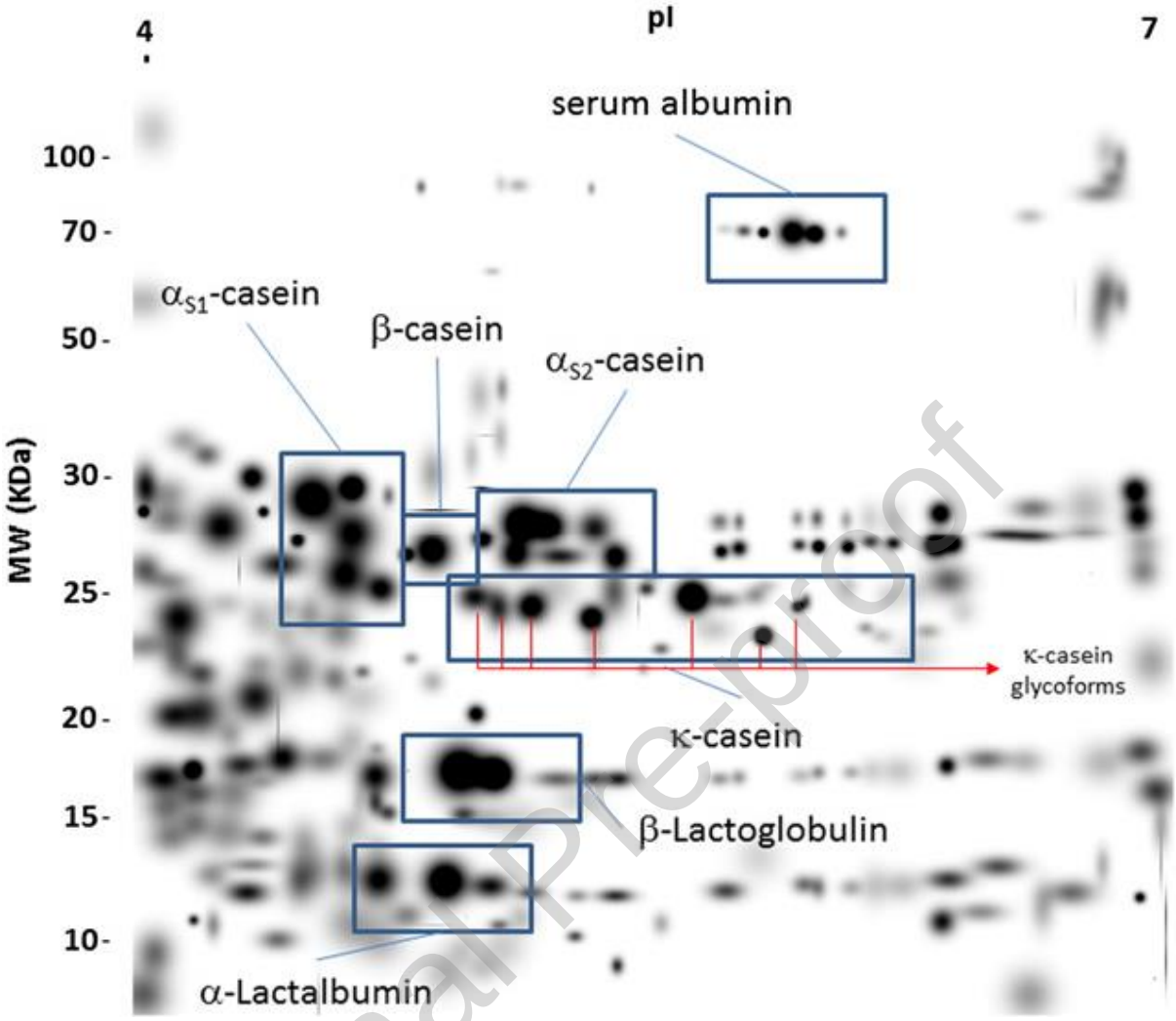


Figure 5: 2-Dimensional electrophoresis of bovine milk proteins. Reproduced from Roncada et al. (2012) with permission from Elsevier.

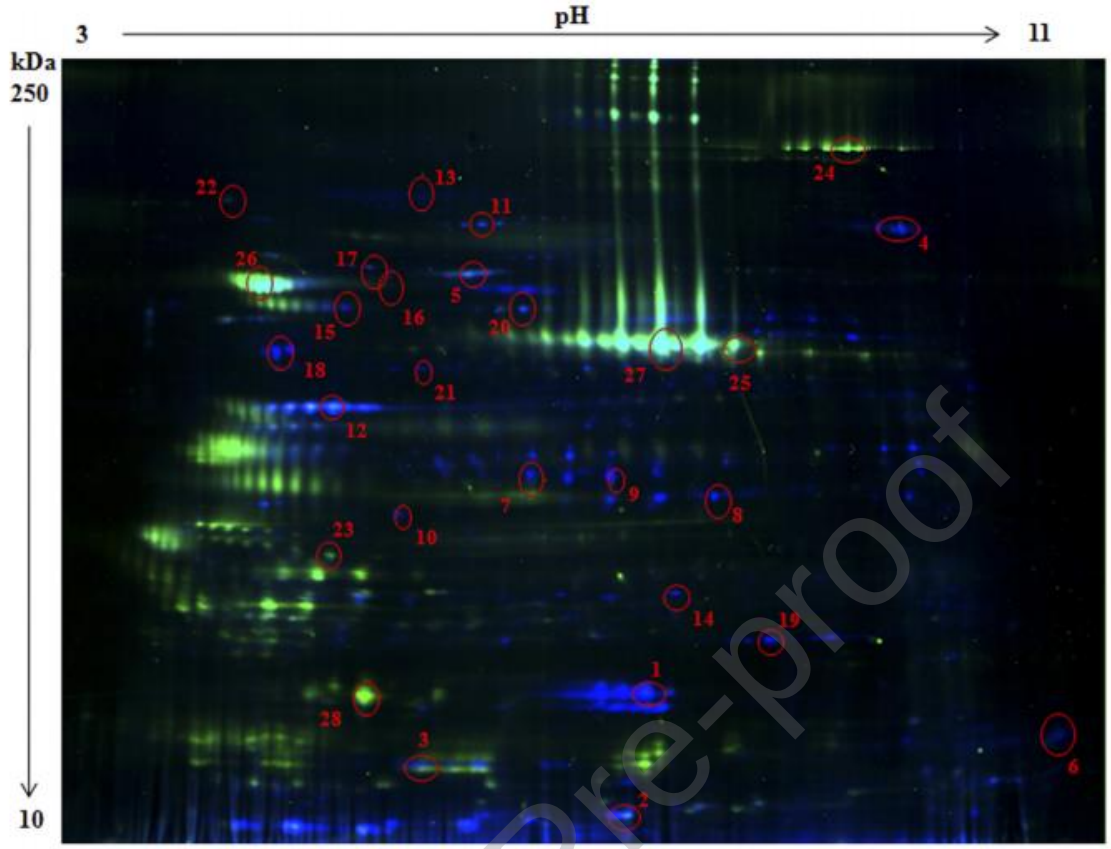


Figure 6: 2-D DIGE of MFGPs from *M. agalactiae*-infected and uninfected sheep. Overlay image of MFGPs extracted from representative infected (blue) and uninfected (green) milk samples. Spots indicate proteins with statistically significant differences in amount among all samples examined. Reproduced from Addis et al. (2011) with creative commons permission.

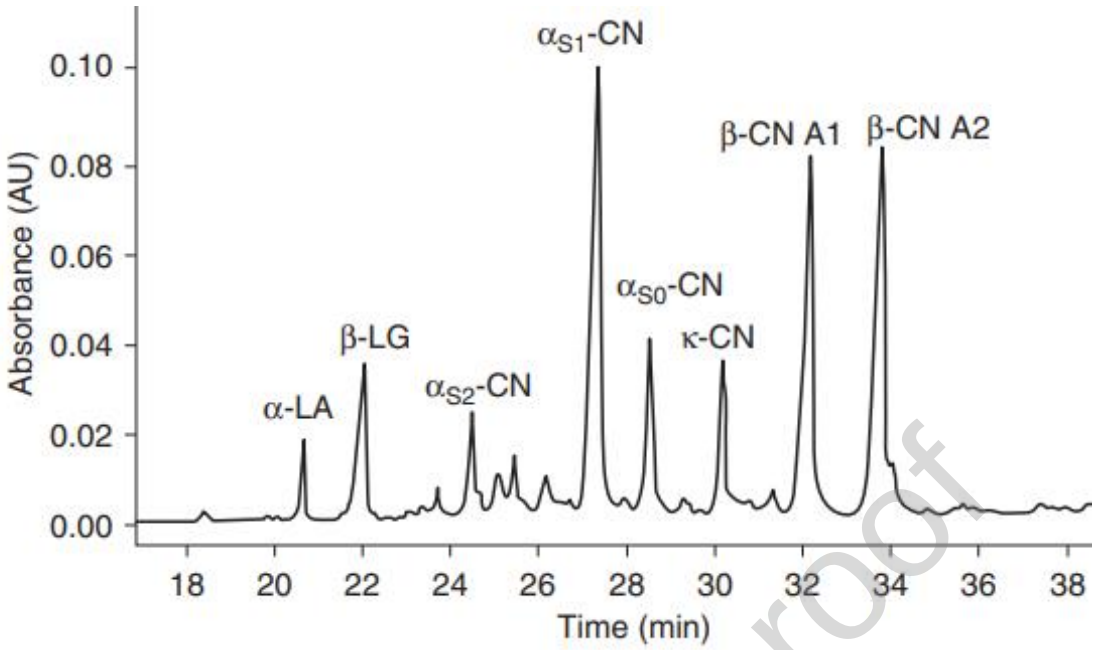


Figure 7: Representative electropherogram of milk proteins using high performance capillary electrophoresis (HPCE). Reproduced from Heck et al. (2008) with permission from Elsevier.

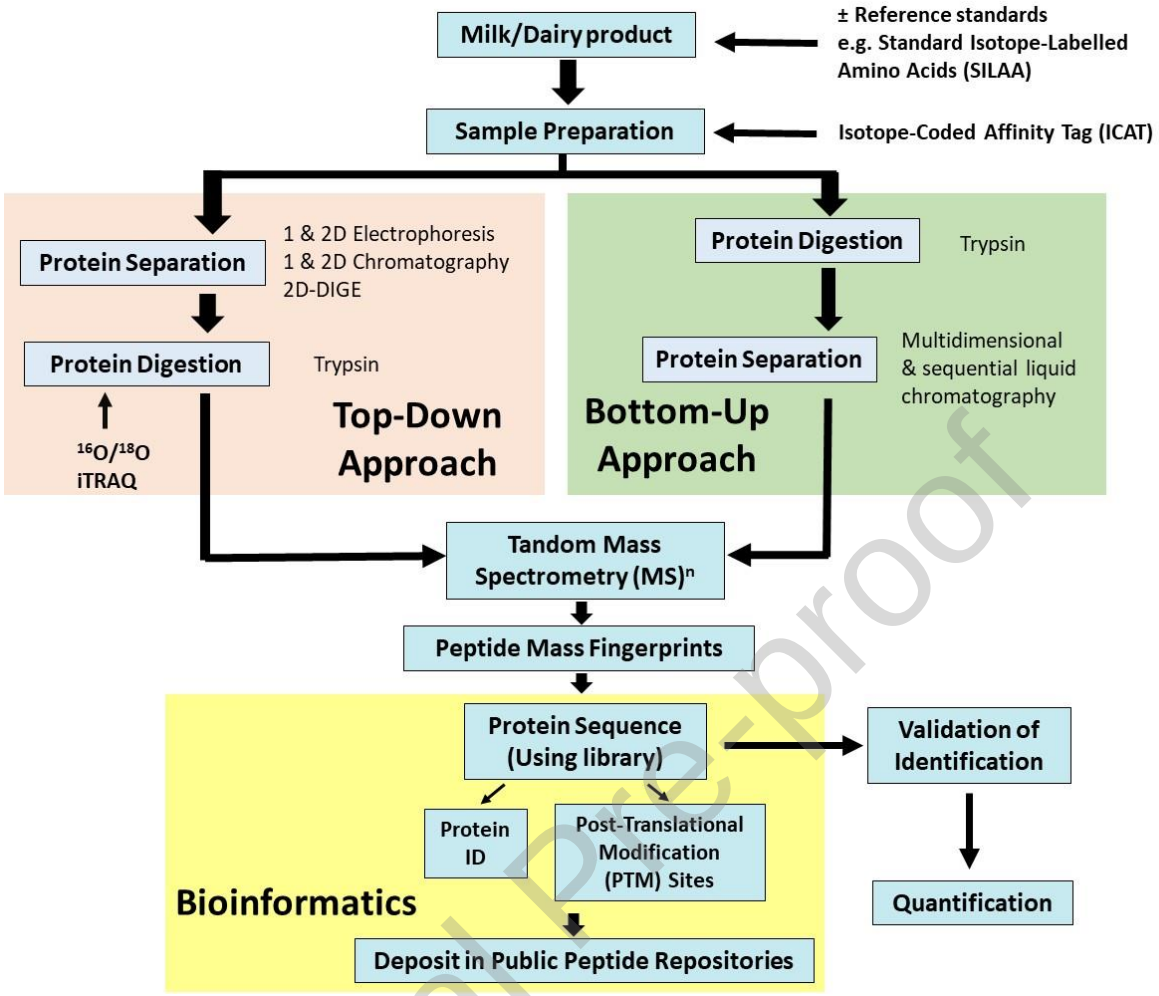


Figure 8: Schematic overview of proteomic analysis methods.

CRediT authorship contribution statement

Santhoshani Warakaulle, Huda Mohamed: Writing – original draft, Writing – review & editing,

Meththa Ranasinghe, Iltaf Shah, Xu Yanyang, Gang Chen, Mutamed M. Ayyash, Delphine Vincent: Writing review & editing, Afaf Kamal-Eldin: Conceptualization, Funding, Writing review & editing.

Declaration of Interest Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Highlights

- Milk protein analysis is carried out through an array of intricate methods.
- Titration and spectrophotometry are utilized to determine total milk proteins.
- Advanced biochemical techniques separate, identify, and quantify specific proteins.
- NMR, X-ray, and mass spectroscopic techniques define the structure of proteins.
- Researchers may opt for the most appropriate technique based on their application.