

# Milk protein and peptide profiles under different heat loads: Molecular analysis

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## Abstract:

Heat treatment induces structural changes in milk proteins, thus affecting the processing suitability of milk powder. Identifying new molecular markers of heat load helps to develop rapid and efficient approaches to milk powder assessment. This study utilized molecular analysis to investigate the impact of thermal treatment on the proteome and peptidome of skim milk.

The skim milk samples were subjected to heat treatment at 70, 80, and 90°C for 30 s, while skim milk maintained at 45°C served as control. Two-dimensional electrophoresis made it possible to separate and evaluate protein structural changes. Peptide identification relied on matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), preceded by trypsinolysis. A combination of Raman spectroscopy and principal component analysis visualized the heat-induced spectral responses to evaluate the potential of Raman spectroscopy for tracing the thermal history of milk powder.

High pasteurization temperature reduced the number of protein fractions. MALDI-TOF MS identified 30 peptides belonging to seven major milk proteins. Peptide fragments of  $\alpha_{s1}$ -casein and  $\beta$ -casein were absent in the control but present in the experimental samples, indicating heat-induced protein transformation. The intermolecular interactions and disruption of the secondary structure of proteins resulted in differences in the isoelectric points. Raman spectroscopy revealed heat-induced changes in the wavenumber ranges of 900–1097, 1328–1437, and 1475–1546  $\text{cm}^{-1}$ , attributed to the transformed carbohydrate and proteomic milk profiles. The principal component analysis made it possible to visualize the differences and identify the regions that make the highest contribution to the response.

Raman spectroscopy proved to be a reliable technique for developing and validating a rapid approach to assess the degree of heat load in milk.

**Keywords:** Skim milk, milk powder, heat treatment, peptide profile, milk proteins, two-dimensional electrophoresis, mass spectrometry, Raman spectroscopy

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## INTRODUCTION

Dairy production is driven by the growing global population and the increasing demand for high-quality food [1]. It is expected to grow by 22% by 2027 [2]. Dairy products have always been an integral part of the traditional Russian diet. Starting in 2015, a decade-long increase in raw milk production reached 34 million tons in 2024 [3], i.e., an increase of 13.7%. As the dairy sector keeps growing, dairy processing plants have to increase production efficiency while ensuring rapid and accurate

quality control [2]. Taking into account this challenge, modern quality control must shift from conventional regulatory indicators to assessing food properties during processing and storage [4]. Milk powder is part of dairy, bakery, confectionery, and other formulations. As such, it requires a comprehensive in-depth analysis of its composition and properties. Skim milk powder is the most in-demand in the food industry. In 2024, the global production of skim milk powder amounted to 150.3 billion US dollars. With an average annual growth rate of 5.8%, it is expected

to exceed 260 billion US dollars by 2034<sup>1</sup>. In Russia, technical modernization and state support also ensure a stable increase in skim milk powder production. From January to October 2025, its volume grew by 11.8% compared to the same period in 2024 and reached 108,700 tons<sup>2</sup>.

As a complex colloidal system of proteins, fats, carbohydrates, minerals, and vitamins, milk requires multidisciplinary analysis. The problem is that its components vary in physicochemical and structural properties, which complicates quality assessment during processing. Proteins are responsible for the allergenicity [5], functional characteristics [6], biological value [7], and technological properties of milk. Their components ( $\alpha$ -,  $\beta$ -, and  $\kappa$ -caseins, whey proteins, immunoglobulins, and enzymes) have disparate properties [8].

Heat treatment is a mandatory process in the milk production chain as it ensures microbiological safety. The processing conditions vary greatly: sterilization, pasteurization, ultra-pasteurization, etc. As a result, the thermal exposure of the milk system components differs significantly [9]. In this regard, studying changes in milk proteins during heating is important for food safety and defines their further technological use [10, 11]. A rapid thermal load test means an equally rapid charting of utilization pathways.

For example, fermented dairy products require high pasteurization temperatures, and the heating mode targets protein structural changes necessary to reach the desired consistency [12]. On the contrary, cheese and cottage cheese obtained by acid-rennet coagulation need a lower degree of whey protein denaturation to obtain a curd with the necessary properties [13].

Global and domestic skim milk powder producers classify it into three main raw-material classes, depending on the whey protein nitrogen index (WPNI). This indicator is determined using the Kjeldahl method and correlates with the degree and time of thermal exposure. For instance, pre-curding processing parameters of 70°C for 15 s yield 6.00 mg WPNI/g of low-heat skim milk powder; 85°C for 60 s or 90–105°C for 30 s result in 1.51–5.99 mg WPNI/g of medium-heat skim milk powder; 90°C for 5 min or 120°C for 1–2 min produce as little as 1.50 mg WPNI/g high-heat skim milk powder [14–16].

Studies of this kind discover new heat load markers to improve the current dairy quality control procedures.

Molecular analysis methods standardize the assessment of heat load effect on dairy raw materials and help to chart further utilization pathways. They are sensitive, accurate, and comprehensive. For instance, Raman spectroscopy is fast and automated. It can be applied to complex dairy systems without special pretreatment or sample destruction.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a multitask molecular analysis method [11]. Other similar methods include various types of electrophoresis [17, 18], IR or Raman spectroscopy for protein analysis [19], and the method of polymerase chain reaction for DNA and RNA studies [20].

Polymerase chain reaction methods are able to detect the adulteration of high-quality dairy products through the addition of cheaper raw materials, e.g., cow's milk instead of goat's or sheep's milk [21, 22]. They also detect residual amounts of pathogenic microorganisms in products that are otherwise undetectable by classical methods [23].

Computer-aided mass spectrometry assesses heat- or storage-induced changes in milk. Losito *et al.* [24] developed a method for assessing the heat treatment based on the degree of lactosylation of whey proteins. Class *et al.* [25] used high-resolution mass spectrometry and custom-designed software to identify eight peptides undergoing changes in ultra-pasteurized milk during storage. The established shelf life corresponded with the actual storage-induced changes. The main changes were observed in peptides related to  $\alpha_{s1}$ -casein,  $\alpha_{s2}$ -casein,  $\beta$ -casein, and  $\beta$ -lactoglobulin. The signal intensity of all these peptides increased together with ultra-pasteurization. Šebela [26] mentioned mass spectrometric approaches for assessing adulteration of milk and dairy products. Kang *et al.* [27] combined two-dimensional electrophoresis and mass spectrometry to determine the optimal processing pathway and detect markers of excessive heat load in dairy products.

Raman spectroscopy also proved effective in assessing the degree of heat load by tracing the conformational changes in milk proteins. These changes are associated with specific amide bands, namely Amide I ( $\sim 1,650\text{ cm}^{-1}$ ) and Amide III ( $\sim 1,250\text{ cm}^{-1}$ ). They serve as signal regions sensitive to thermal effect [19]. The position and shape of the amide bands tell volumes about the secondary structure of proteins and even the share of individual structural components, e.g.,  $\alpha$ -helices and  $\beta$ -structures. Raman spectroscopy was reported to be able to identify 10% of  $\alpha$ -helices and a significant percentage of irregular  $\beta$ -structures in caseins [28]. Zhang *et al.* [29] applied Raman spectroscopy to the aggregation of whey proteins under heat load with and without pectin (0.015–0.20 wt.).

Li *et al.* [30] used a specific proteomic approach based on liquid chromatography with mass spectrometry (LC-MS/MS) to detect changes in whey protein systems heated to  $\geq 90^\circ\text{C}$ . Whey protein isolates demonstrated less protein oxidation compared to pure proteins ( $\alpha$ -lactalbumin  $>$   $\beta$ -lactoglobulin) and a mixed-protein whey model. Cysteine, methionine, and tryptophan residues in  $\alpha$ -lactalbumin were found to be more susceptible to oxidation.

Meltretter *et al.* [31] studied markers of non-thermal and thermal exposure in dairy raw materials using ultra-high-performance liquid chromatography with mass spec-

<sup>1</sup> Skimmed milk powder market size and share 2025–2034. Global Market Insights. [cited 2026 Jan 20]. (In Russ.) Available from: <https://www.gminsights.com/ru/industry-analysis/skimmed-milk-powder-market>

<sup>2</sup> The production of skimmed milk powder increased by 11.8%. Milknews. [cited 2026 Jan 20]. (In Russ.) Available from: <https://milknews.ru/analitika-rinka-moloka/rinok-moloka-v-Rossii/som-analitika-noyabr-2025.html>

trometry and multiple reaction monitoring. Lactosyl lysine proved to be a marker of low-heat treatment while *N*<sup>6</sup>-carboxymethyl lysine, *N*-terminal ketoamide, and asparagine deamidation marked more intense thermal stress.

To rationally select the degree of heat treatment and identify markers for its monitoring, Lu *et al.* [32] used the proteomic method of LC-MS/MS to study lactosylated proteins subjected to various thermal modes. As a result, they detected a total of 123 lactosylated lysines in 65 proteins. Between 75 to 130°C, their number increased smoothly and moderately but soared when the temperature reached 140°C. More than half of the lysine residues were lactosylated in  $\kappa$ -casein,  $\beta$ -lactoglobulin, and  $\alpha$ -lactalbumin. The fact that K<sub>14</sub> lactosylated in  $\beta$ -lactoglobulin allowed the authors to tell between pasteurized and ultra-pasteurized milk: lactosylated lactotransferrin indicated medium-heat processing.

Therefore, the effect of heat load on the dairy system is a popular research topic approached using various molecular analysis methods. Yet, no immediate method exists for determining this parameter.

This study evaluated the feasibility of using Raman spectroscopy to detect changes in the proteomic profile of pasteurized skim milk under varying heat loads.

## STUDY OBJECTS AND METHODS

**Study objects.** To assess the effect of heat load on skim milk proteins, we heated the experimental samples to 70, 80, and 90°C with a holding time of 30 s. The sample maintained at 45°C served as control (Table 1).

The experimental samples were produced using the equipment from the Shared Use Center at the All-Russian Dairy Research Institute, Moscow (Fig. 1). Raw milk (pH 6.67) was separated using an Alfa Laval laboratory separator (Alfa Laval Group, Sweden) and heated using a pilot pasteurization and cooling unit (1 L/h) designed at the All-Russian Dairy Research Institute.

The protein profile of the resulting skim milk samples was analyzed by two-dimensional electrophoresis.

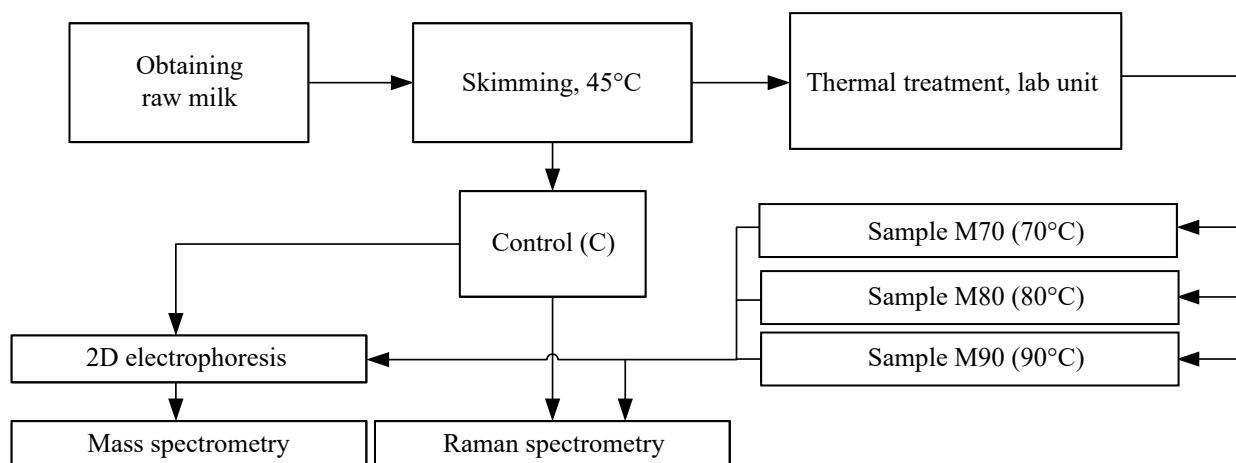
The peptide profile identification was performed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) preceded by the trypsinolysis of protein spots. Raman spectroscopy was involved to be assessed for prospects in profiling milk powder by its thermal history.

**Two-dimensional electrophoresis.** Two-dimensional electrophoresis involved isoelectric focusing in ampholine buffer in line with the method described in [33]. The gels were stained with silver nitrate to visualize protein spots. The control sample was represented by a set of 14 highly purified recombinant proteins (10–200 kDa, PageRuler™ Unstained Protein Ladder #SM0661; Fermentas, USA) to determine protein molecular weights.

**Peptide profiling by MALDI-TOF MS.** To identify proteins, we excised individual fractions from two-dimensional electropherogram gels to grind them and subject them to trypsinolysis as in [34]. The resulting protein-containing gel fragments were incubated for 45 min in hydrolysis buffer while cooling in an ice bath. The buffer consisted of 50 mM NH<sub>4</sub>HCO<sub>3</sub>; 5 mM CaCl<sub>2</sub>; 12.5 ng/μL trypsin (V5551; Promega, USA; activity 10,000 U/mg). After removing the supernatant and adding 5–10 μL of enzyme-free buffer, we performed hydrolysis at 37°C for 3 h, followed by enzyme inactivation with 0.1% trifluoroacetic acid solution. The peptide tests involved an Ultraflex time-of-flight mass spectrometer (Bruker, Germany) with a UV laser (336 nm) in the positive ion mode at 500–8,000 Da with trypsin autolysis for calibration.

**Table 1** Samples' description

| Sample | Description                                 |
|--------|---------------------------------------------|
| C      | Control: skim milk, no pasteurization, 45°C |
| M70    | Pasteurized skim milk, 70°C, 30 s           |
| M80    | Pasteurized skim milk, 80°C, 30 s           |
| M90    | Pasteurized skim milk, 90°C, 30 s           |



**Figure 1** Experimental design

**Mass spectrometry processing.** The obtained tryptic peptide mass spectra were analyzed using Mascot Peptide Fingerprint (Matrix Science, USA), with an  $MH^+$  weight determination accuracy of 0.01%. The calculations relied on the data published by the National Center for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov/>).

All proteomic studies were performed on the premises of the Federal Research Center for Industrial Biotechnology, Russian Academy of Sciences, Moscow.

**Amino acid sequence evaluation.** This part of the research involved sequences available in the UniProt database, UK, for  $\alpha$ -lactalbumin (P00711) and lysozyme C (Q6B411). The pairwise alignment was performed using the ClustalW algorithm within Jalview (UK) with default parameters.

**Raman spectroscopy.** Raman spectra were obtained using an EnSpectr R785 portable Raman spectrometer (Spektr-M, Russia) at  $250\text{--}2700\text{ cm}^{-1}$ . The measurements were triplicated at five different acquisition points for each sample. The laser wavelength was 785 nm. The resulting spectral profiles made it possible to evaluate the heat-induced spectral response of the milk system. The graphical representation and subsequent analysis relied on averaged spectra for each sample group.

**Spectral data processing.** The averaged spectral dataset was processed using Principal Component Analysis (Python) in Numpy, Pandas, and Sklearn. A Z-transform (standardization) assigned each feature a zero mean and a unit variance. The analysis was per-

formed at  $900\text{--}1700\text{ cm}^{-1}$ ; the spectrum was divided into fixed-width regions. Principal component analysis served as an exploratory visualization tool for heat-induced spectral changes (MinMaxScaler, 0 to 1).

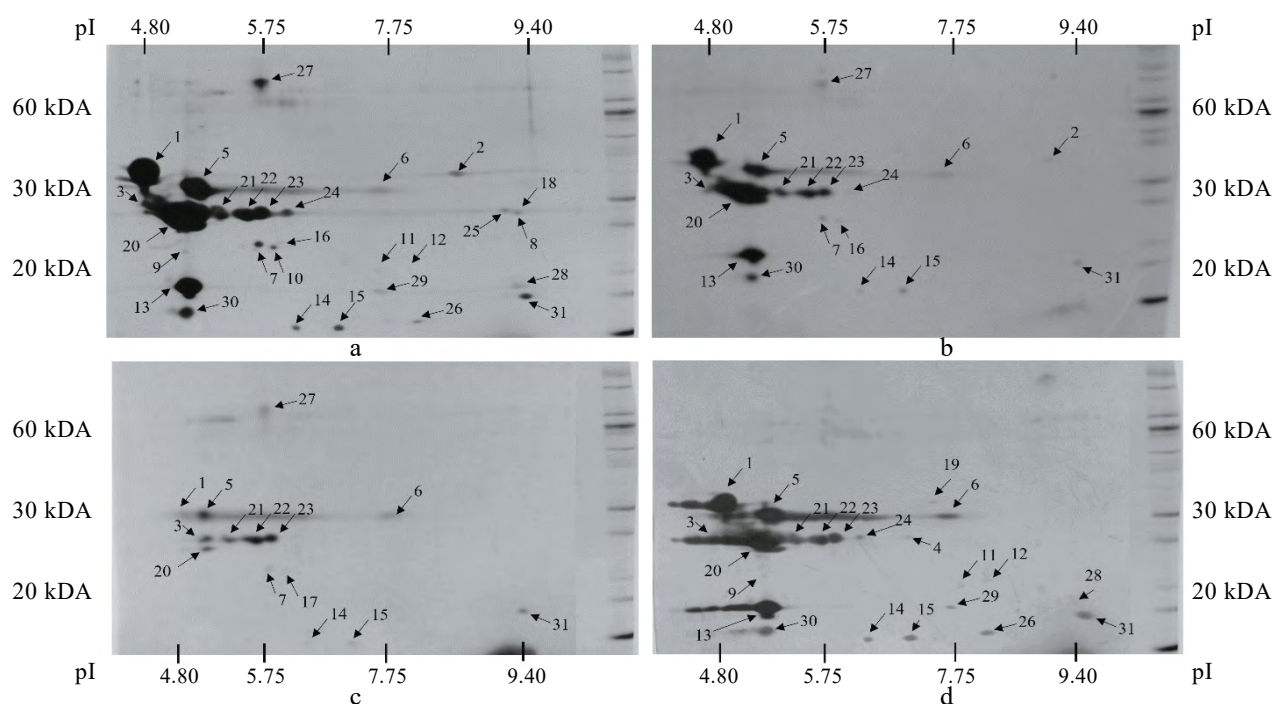
## RESULTS AND DISCUSSION

### Electrophoretic separation and peptide profiles.

Two-dimensional electrophoresis yielded a set of protein spots of milk casein fraction and whey proteins (Fig. 2). The casein fraction included isoforms of  $\alpha_{s1}$ -casein and  $\beta$ -casein,  $\kappa$ -casein, and heat-induced protein spots. Whey proteins included bovine serum albumin,  $\alpha$ -lactalbumin, and  $\beta$ -lactoglobulin.

The casein fraction of milk proteins remained clearly visible in the electropherograms even after the heat load because caseins are more resistant to thermal treatment than whey proteins [35]. These milk proteins are characterized by a protein band and spots that appear for a specific isoform of the same molecular weight at different isoelectric points. Such distribution may be attributed to post-translational modifications or non-enzymatic changes (e.g., lactosylation) caused by storage and heating [36].

Whey proteins are heat-labile: heat-induced hydrophobicity occurs at  $70\text{--}90^\circ\text{C}$ , making them form insoluble coagulates or even precipitate [37]. The electropherogram fragments demonstrated a significant decrease in the color intensity in the regions related to bovine serum albumin,  $\alpha$ -lactalbumin, and  $\beta$ -lactoglobulin. The most significant decrease in the number and color intensity of protein fragments belonged to sample M80 ( $80^\circ\text{C}$ ), compared to samples M70 ( $70^\circ\text{C}$ ) and M90 ( $90^\circ\text{C}$ ).



**Figure 2** Two-dimensional electropherograms of skim milk samples: a – control ( $45^\circ\text{C}$ ); b – M70 ( $70^\circ\text{C}$ ); c – M80 ( $80^\circ\text{C}$ ); and d – M90 ( $90^\circ\text{C}$ ). Protein assignment was determined using MALDI-TOF MS after trypsinolysis: 1–4 –  $\alpha_{s1}$ -casein; 5, 6 –  $\alpha_{s2}$ -casein; 7 –  $\beta$ -casein (isoform X1); 8–19 –  $\beta$ -casein (isoform X2); 20–26 –  $\kappa$ -casein; 27 – bovine serum albumin; 28–30 –  $\beta$ -lactoglobulin; 31 – not determined

This result may be attributed to the effect of sodium dodecyl sulfate on the aggregates formed between  $\beta$ -lactoglobulin and  $\kappa$ -casein [38]. At 70–80°C, the  $\beta$ -lactoglobulin protein globule partially unfolds to release the thiol group and form bonds with  $\kappa$ -casein. The intensity of this process depends on the temperature and ionic composition of the milk matrix. In addition, heat treatment may transform non-covalent bonds between whey proteins and casein fractions into covalent ones [39, 40]. The surface of the resulting protein complex is hydrophobic and has a small number of free sulfhydryl groups, which hinders the interaction with sodium dodecyl sulfate [41]. A temperature of  $\geq 90^\circ\text{C}$  changes the balance of non-covalent and covalent bonds in the aggregates of  $\beta$ -lactoglobulin and  $\kappa$ -casein. This phenomenon mitigates spatial limitations during protein-reagent interactions, facilitating more efficient peptide diffusion and distribution within the polyacrylamide gel matrix [38].

Such changes in milk proteins affect the technological properties of milk powder and may ultimately limit its use as raw material. As the share of native  $\alpha$ -lactalbumins and  $\beta$ -lactoglobulins goes down, the rennetability deteriorates [13]. On the other hand, the formation of  $\beta$ -lactoglobulin –  $\kappa$ -casein protein complexes can positively improve the structural characteristics of fermented milk products: curd firmness increases while syneresis decreases [42, 43].

Ichimura *et al.* [42] used the methods of scanning and transmission electron microscopy to study the quality indicators of yogurt produced from milk pasteurized at 95 and 130°C. The samples obtained from high-heat milk demonstrated a better curd structure owing to the interaction between  $\beta$ -lactoglobulin and  $\kappa$ -casein.

The capacity for acid or acid-rennet coagulation is not the only important technological property of milk. Solubility is another key indicator for milk powder. Its rate depends on the degree of transformation of the protein matrix [7]. These transformations correlate with poor solubility, emulsifying efficiency, and foamability of milk powder. Extensive milk processing increases the number of modified and aggregated forms of protein in the finished product. These forms spoil the technological properties and shorten the shelf life of milk powder. Changes in the ratio of undenatured and denatured milk proteins were reported to affect the solubility index and stability of reconstituted milk, as well as the properties of acid and rennet curd [35, 44].

The mass spectrometry of the peptide profile, performed after trypsinolysis, revealed 30 amino acid sequences belonging to seven major milk proteins:  $\alpha_{\text{S1}}$ -casein (4);  $\alpha_{\text{S2}}$ -casein (2);  $\beta$ -casein, isoform X1 (1);  $\beta$ -casein, isoform X2 (12);  $\kappa$ -casein (7); bovine serum albumin (1);  $\beta$ -lactoglobulin (3) (Table 2). The prevailing share of  $\alpha$ - and  $\beta$ -casein fragments was consistent with the data reported in [45, 46]. These studies in the peptidomics of milk and dairy products employed high-resolution liquid chromatography with mass spectrometry profiling, which related most peptides to casein fractions. Amino acid sequences of whey proteins

were less frequent. This feature of the milk system may be associated with the greater susceptibility of casein to plasmin in raw milk.

The matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) data demonstrated the prevalence of casein peptide fragments (26 peptides) over whey protein fragments (4 peptides). This imbalance is typical of dairy systems since casein fractions constitute 80% of the total protein content in milk [47]. On the other hand, it can be explained by the properties of casein micelles, which are thermostable protein elements of the dairy system. When heated, they do not denature but undergo structural changes and mediate the appearance of new protein spots, i.e., spots do not disappear during electrophoretic separation but their forms or fragments are redistributed [8].

We identified three amino acid sequences for  $\alpha_{\text{S1}}$ -casein in the control sample. One of them (2) had significant deviations in the isoelectric point value: the calculated value was 4.98 pH units while the experimental one was 8.8 pH units.  $\alpha_{\text{S1}}$ -casein is characterized by phosphorylation at several amino acid residues [48]. Fraction 2 resulted from the loss of phosphate groups, which shifted the isoelectric point to the basic region, destabilizing the molecule and leading to denaturation. Sample M90 (90°C) had fragment 4, identified as  $\alpha_{\text{S1}}$ -casein. Its isoelectric point was 6.70 pH units, which also exceeded the calculated value. This result may be associated with the appearance of new amino acid sequences caused by protein cross-linking [49].

Phosphorylation at 10–13 residues was also characteristic of  $\alpha_{\text{S2}}$ -casein, which means that the calculated isoelectric point (8.54 pH units) did not coincide with the experimental value, as was the case for  $\alpha_{\text{S1}}$ -casein. The main peptide fragment (fragment 5) of  $\alpha_{\text{S2}}$ -casein occurred at the isoelectric point of 5.00 pH units because phosphorylation shifted the isoelectric point to the acidic region. Fragment 6 with the isoelectric point in the slightly alkaline region may also be due to partial loss of phosphate groups [50].

For  $\beta$ -casein, the fractions separated into two isoforms, and isoform X1 had only one fragment, fragment 7. Isoform X2 demonstrated several major fractions and a number of fragments. This uneven distribution is probably explained by the fact that isoform X1 belongs to  $\beta$ -casein A1 while isoform X2 belongs to a more common type,  $\beta$ -casein A2 [51]. Peptide 13 was closest to the calculated isoelectric point value (5.34 pH units) and molecular weight (24.3 kDa). The lower electrophoretic activity of other fragments can be explained by the amino acid composition of  $\beta$ -casein: its proline residues prevented the polypeptide chain from compact folding [37]. This factor probably destabilized  $\beta$ -casein molecules upon heating and caused the fractions to disappear. The smaller molecular weight fragments could be explained by the activity of plasmin, which is known to cleave  $\beta$ -casein into fragments while retaining its activity even after heat treatment [45]. Proteolytic cleav-

**Table 2** Peptide profile of pasteurized skim milk samples: Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

| Fragment | Protein                     | NCBI code      | pI, pH units |      | MW, kDa |      | Peptides, units* | Sequence coverage, %* |
|----------|-----------------------------|----------------|--------------|------|---------|------|------------------|-----------------------|
|          |                             |                | calc.        | exp. | calc.   | exp. |                  |                       |
| 1        | $\alpha_{s1}$ -casein       | NP_851372.1    | 4.98         | 4.75 | 24.5    | 36.0 | 8                | 38                    |
| 2        |                             |                |              | 8.80 |         | 35.0 | 7                | 33                    |
| 3        |                             |                |              | 4.90 |         | 30.0 | 5                | 31                    |
| 4        |                             |                |              | 6.70 |         | 29.5 | 4                | 26                    |
| 5        | $\alpha_{s2}$ -casein       | NP_776953.1    | 8.54         | 5.00 | 26.0    | 34.0 | 10               | 45                    |
| 6        |                             |                |              | 7.70 |         | 34.0 | 3                | 19                    |
| 7        | $\beta$ -casein, isoform X1 | XP_010804480.3 | 5.53         | 5.70 | 25.2    | 24.5 | 1                | 8                     |
| 8        | $\beta$ -casein, isoform X2 | XP_015327157.2 | 5.36         | 9.20 | 24.3    | 29.0 | 4                | 23                    |
| 9        |                             |                |              | 4.95 |         | 27.0 | 2                | 7                     |
| 10       |                             |                |              | 5.90 |         | 24.0 | 5                | 18                    |
| 11       |                             |                |              | 7.70 |         | 25.0 | 3                | 12                    |
| 12       |                             |                |              | 7.90 |         | 23.0 | 3                | 12                    |
| 13       |                             |                |              | 5.00 |         | 23.0 | 2                | 15                    |
| 14       |                             |                |              | 6.20 |         | 13.5 | 1                | 8                     |
| 15       |                             |                |              | 6.70 |         | 13.5 | 4                | 18                    |
| 16       |                             |                |              | 5.90 |         | 27.0 | 5                | 22                    |
| 17       |                             |                |              | 5.90 |         | 27.0 | 2                | 15                    |
| 18       |                             |                |              | 9.20 |         | 29.0 | 1                | 8                     |
| 19       |                             |                |              | 7.50 |         | 36.0 | 3                | 12                    |
| 20       | $\kappa$ -casein            | NP_776719.1    | 6.30         | 4.95 | 21.2    | 29.0 | 1                | 5                     |
| 21       |                             |                |              | 5.30 |         | 29.0 | 7                | 35                    |
| 22       |                             |                |              | 5.50 |         | 29.0 | 9                | 37                    |
| 23       |                             |                |              | 5.80 |         | 29.0 | 9                | 37                    |
| 24       |                             |                |              | 6.00 |         | 29.0 | 9                | 37                    |
| 25       |                             |                |              | 9.10 |         | 29.5 | 7                | 37                    |
| 26       |                             |                |              | 8.00 |         | 13.5 | 4                | 30                    |
| 27       | Bovine serum albumin        | NP_851335.1    | 5.82         | 5.50 | 69.3    | 67.0 | 23               | 42                    |
| 28       | $\beta$ -lactoglobulin      | NP_776354.2    | 4.93         | 9.20 | 19.8    | 20.0 | 13               | 72                    |
| 29       |                             |                |              | 7.70 |         | 7.70 | 1                | 11                    |
| 30       |                             |                |              | 5.00 |         | 20.0 | 1                | 8                     |
| 31       | n.d.                        | –              | –            | 9.40 | –       | 15.5 | 6                | 19                    |

pI – protein's isoelectric point, pH units; calc. – calculated value; exp. – experimentally determined value (2D electrophoresis); MW – molecular weight, kDa; \* – peptide profile (matrix-assisted laser desorption ionization time-of-flight mass spectrometry); n.d. – (not detected) bioinformatics processing identified the fragment as  $\alpha$ -lactalbumin, which cannot correspond to the experimentally determined isoelectric point. NCBI – National Center for Biotechnology Information

age can also explain the appearance of fragment 19 in Sample M90 (90°C) since heat-induced unfolding of the casein micelle opens up new sections of the polypeptide chain for cleavage by plasmin.

$\kappa$ -Casein demonstrated a number of peptide fragments with different isoelectric points but the same molecular weight, which exceeded the calculated one. The isoelectric point values shifted relative to the calculated value, both towards the acidic and towards the basic pH range.  $\kappa$ -Casein glycosylates with sialic acids; therefore, the dispersion of  $\kappa$ -casein fractions can be explained by the differences in glycation of amino acid residues [52, 53]. A greater number of carbohydrate molecules attached to the protein molecule shifts the isoelectric point towards the acidic region.  $\kappa$ -Casein can also form complexes with whey protein peptides, which appear as new fractions [37].

We identified three amino acid sequences for  $\beta$ -lactoglobulin, confirming the nature of the protein change upon a heat load of  $\leq 80^\circ\text{C}$ . Globular unfolding leads to thiol group exposure, facilitating protein-to-protein interactions via the newly accessible groups [54].

These discrepancies between the calculated and experimental isoelectric points may be due to some structural changes induced by thermal treatment, e.g., the aggregation of protein molecules, the formation of covalent cross-links during protein-to-protein interactions, etc. Such modifications of the proteomic profile affect the charge of protein substances, ultimately changing the isoelectric point [49].

Fragment 31 proved to be another feature of the mass spectrometric peptide profiling. The protein was identified as  $\alpha$ -lactalbumin via bioinformatic processing

of the mass spectra. However, the isoelectric point of  $\alpha$ -lactalbumin is 4.8 pH units whereas the value of the obtained fragment was 9.4 pH units. The evolutionary and structural homologue of  $\alpha$ -lactalbumin is lysozyme C (LyzC) [55].  $\alpha$ -Lactalbumin consists of 142 amino acid residues while lysozyme C has 148. Their molecular masses are very close: 14.2 and 14.4 kDa, respectively. The calculated value of the isoelectric point of lysozyme C is 9.87 pH units, which is consistent with the obtained values. Probably, the error in the bioinformatics processing of the mass spectra occurred due to the similar amino acid sequences of  $\alpha$ -lactalbumin and lysozyme C. The amino acid sequences of  $\alpha$ -lactalbumin and lysozyme C (Fig. 3) revealed a similarity of approximately 40%, which was consistent with the data reported in [55]. The similar amino acid composition of these proteins stem from a common evolutionary ancestor of the coding genes.

The amino acid sequence demonstrated some common features, e.g., the arrangement of cysteine residues. In  $\alpha$ -lactalbumin, they were located at positions 25, 47, 80, 92, 96, 110, 130, and 139. In lysozyme C, they were at positions 24, 48, 83, 95, 99, 113, 134, and 146. This shared feature explains the structural similarity of the proteins. However, since the conserved regions are different, the biochemical properties are different, too.

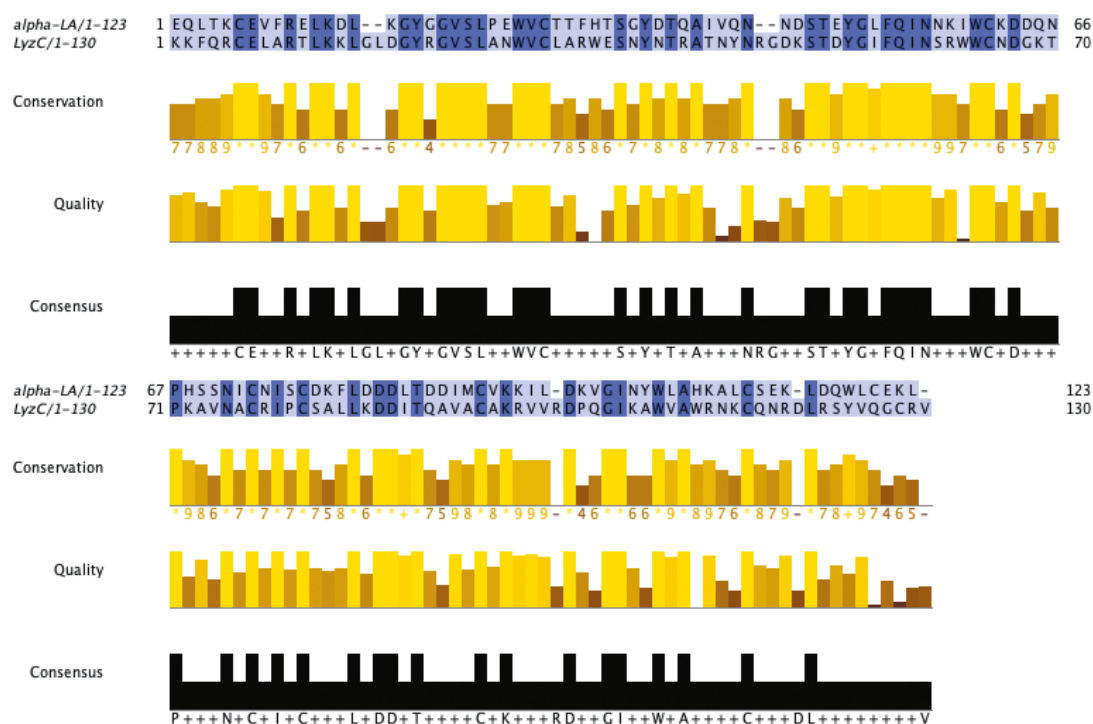
Lysozyme C is responsible for antibacterial properties. This protein exhibits hydrolytic activity against the  $\beta$ -1,4-glycosidic bond in the peptide glycan of the bacte-

rial cell wall. As a result, it has an active site that consists of catalytically essential amino acids – glutamine (Glu35) and asparagine (Asn52). Lysozyme C is rich in lysine (18 residues) and arginine (17 residues), which means that its isoelectric point shifts toward the base region. At pH 6.6–6.8, the protein has a positive charge, which facilitates the binding of a negatively charged substrate [56].

$\alpha$ -Lactalbumin starts functioning already during lactose synthesis in the mammary gland as a regulator of the lactose synthase complex. For the protein-to-protein interaction between  $\alpha$ -lactalbumin and galactosyl-transferase to be effective,  $\alpha$ -lactalbumin must be bound to a calcium ion. Calcium is structurally and regulatory essential as it stabilizes the native conformation of  $\alpha$ -lactalbumin. Thus,  $\alpha$ -lactalbumin has a calcium-binding domain that consists of Asp82, Asp84, Asp87, Glu88, and Asp81 residues, which form a  $\text{Ca}^{2+}$  coordination sphere [57].

With only 19% sequence coverage of fragment 31 (Table 2), the mass-spectrometry regions of the  $\alpha$ -lactalbumin and lysozyme C sequences overlap. However, these proteins behave differently when separated by two-dimensional electrophoresis because lysozyme C is fortified with positively charged amino acid residues.

Table 3 illustrates the electrophoretic changes in the proteomic profile of skim milk and the identified peptide profile, thus describing the pasteurization-induced transformations in milk proteins.



**Figure 3** Amino acid sequences:  $\alpha$ -lactalbumin vs. lysozyme. Conservation – column height indicates the degree of conservation at each position, where higher columns represent highly conserved residues; Quality – an indicator of alignment reliability at each specific position; Consensus – the consensus sequence, where letters represent identical residues while “+” indicates conservative substitutions; Numbering – labels (1, 67, 71) denote amino acid positions within the  $\alpha$ -lactalbumin and lysozyme C sequences

Such changes indicate a whole complex of heat-induced processes: partial protein denaturation, aggregation of  $\beta$ -lactoglobulin with  $\kappa$ -casein, possible dephosphorylation of casein fractions, and the appearance of plasmin proteolysis products. Other studies [7, 8] reported similar effects: pasteurization and ultra-pasteurization reduce the proportion of soluble whey proteins in milk, inducing the formation of casein derivative fractions.

According to the mass spectrometry, the control sample (no heat load) demonstrated the most diverse peptide composition (28 protein spots).

As heat load increased, the number of peptides decreased. Sample M90 (90°C) contained some fractions that were absent from lower-heat samples M70 and M80. It even developed some completely new fragments (4 and 19), belonging to  $\beta$ -casein (isoform X2) and  $\alpha_{s1}$ -casein, respectively. The  $\alpha_{s1}$ -casein fragment probably separated during the heat-induced destabilization of casein micelle. In addition, casein micelles could lose phosphates, shifting the isoelectric point toward a more alkaline region [48].

**Table 3** Electrophoretic profile of protein fragments in pasteurized skim milk

| Fragment | Control | M70<br>(70°C) | M80<br>(80°C) | M90<br>(90°C) |
|----------|---------|---------------|---------------|---------------|
| 1        | +       | +             | +             | +             |
| 2        | +       | +             | –             | –             |
| 3        | +       | +             | +             | +             |
| 4        | –       | –             | –             | +             |
| 5        | +       | +             | +             | +             |
| 6        | +       | +             | +             | +             |
| 7        | +       | +             | +             | –             |
| 8        | +       | –             | –             | –             |
| 9        | +       | –             | –             | +             |
| 10       | +       | –             | –             | –             |
| 11       | +       | –             | –             | +             |
| 12       | +       | –             | –             | +             |
| 13       | +       | +             | –             | +             |
| 14       | +       | +             | +             | +             |
| 15       | +       | +             | +             | +             |
| 16       | +       | +             | –             | –             |
| 17       | –       | –             | +             | –             |
| 18       | +       | –             | –             | –             |
| 19       | –       | –             | –             | +             |
| 20       | +       | +             | +             | +             |
| 21       | +       | +             | +             | +             |
| 22       | +       | +             | +             | +             |
| 23       | +       | +             | +             | +             |
| 24       | +       | +             | –             | +             |
| 25       | +       | –             | –             | –             |
| 26       | +       | –             | –             | +             |
| 27       | +       | +             | +             | –             |
| 28       | +       | –             | –             | +             |
| 29       | +       | –             | –             | +             |
| 30       | +       | +             | –             | +             |
| 31       | +       | +             | +             | +             |

The fragments with lower electrophoretic activity could be explained by the amino acid composition of  $\beta$ -casein: it includes proline residues that prevent the polypeptide chain from compact folding [37]. This factor destabilizes  $\beta$ -casein molecules during heating, and the fractions disappear. Fragments with lower molecular weight are explained by the activity of plasmin, which cleaves  $\beta$ -casein and retains its activity under heat load [45]. This factor also explains fragment 30 in sample M90 (90°C): the heat-induced change in the casein micelle's quaternary structure renders the polypeptide chain more susceptible to plasmin cleavage.

Sample M70 (70°C) was closest in protein profile to the control sample. It lost the following protein fragments:  $\beta$ -casein, isoform X2 (8, 9, 10, 11, 12, 16, 18);  $\kappa$ -casein (25, 26);  $\beta$ -lactoglobulin (28, 29). Such peptide profile of milk pasteurized at 70°C was consistent with the results reported in [58]: moderate heat loads induce the denaturation of whey proteins but cause no massive loss of soluble forms and trigger no new high-molecular forms.

Sample M80 (80°C) lacked the following fragments:  $\alpha_{s1}$ -casein (2);  $\beta$ -casein, isoform X2 (8, 9, 10, 11, 12, 13, 16, 18);  $\kappa$ -casein (24, 25, 26);  $\beta$ -lactoglobulin (28, 29, 30). However, it had fragment 17 ( $\beta$ -casein, isoform X2). At 80°C,  $\beta$ -lactoglobulin denatured faster as evidenced by the disappearance of protein spots. Other changes involve intermolecular interactions with  $\kappa$ -casein and the formation of protein aggregates with altered electrophoretic mobility [59].

Sample M90 (90°C) demonstrated the most severe changes in the peptide profile. It lost a significant number of milk protein fragments: bovine serum albumin (27);  $\alpha_{s1}$ -casein (2);  $\beta$ -casein, isoform X1 (7);  $\beta$ -casein, isoform X2 (8, 10);  $\kappa$ -casein (25). Paulsson & Dejmeek [60] reported the disappearance of fragment 27 when they used differential scanning calorimetry to establish the protein denaturation temperature (64°C). Lysozyme C (fragment 31) is more resistant to heat load than  $\alpha$ -lactalbumin. Consequently, as other fragments of whey proteins disappear under heat load, fragment 31 persists under all heat modes. Moriyama *et al.* [61] compared the structural changes in lysozyme C and  $\alpha$ -lactalbumin. Lysozyme C retained a stable secondary structure at  $\geq 60^\circ\text{C}$  whereas  $\alpha$ -lactalbumin lost its native conformation as its protein globule unfolded.

The combined two-dimensional electrophoresis and mass spectrometry yielded highly informative results in relation to milk proteomic profile transformations. However, these methods are so labor-intensive and laboratory-oriented that it limits their application as a rapid analysis of heat-induced changes in the milk matrix, making Raman spectroscopy the most likely candidate.

**Spectral analysis of pasteurized milk samples.** Thermal treatment induces a wide range of chemical reactions in milk, most notably protein denaturation, intermolecular cross-linking, and the Maillard reaction [62]. Raman spectroscopy is highly sensitive to subtle changes in organic molecules, which makes it a promising method for assessing the effects of heat load on milk [63]. We subjected the Raman spectrum to mathematical processing in the wave region that corresponded with milk proteins (900–1700  $\text{cm}^{-1}$ ).

It revealed several zones associated with changes in milk: 900–1097; 1328–1437; and 1475–1546  $\text{cm}^{-1}$  (Fig. 4b).

He *et al.* [63] mentioned using the peak at  $\sim 1085 \text{ cm}^{-1}$  as a marker of lactose in milk because it reflects the vibrations of C-O-H chemical bonds in disaccharide. Similarly, Vaskova & Buckova [64] referred to the band at  $\sim 1087 \text{ cm}^{-1}$  in the Raman spectrum of milk to describe the vibrational vibrations of C-O-H bond in lactose. Reinert *et al.* [65] reported the spectral range of 500–1225  $\text{cm}^{-1}$  as reflecting the changes in milk proteins, including melanoidin formation. They also associated the band at  $\sim 879 \text{ cm}^{-1}$  with changes in proteins, in particular, the indole ring of tryptophan. Therefore, the spectral range of 900–1097  $\text{cm}^{-1}$  marks lactose transformations and a weak Maillard reaction [66].

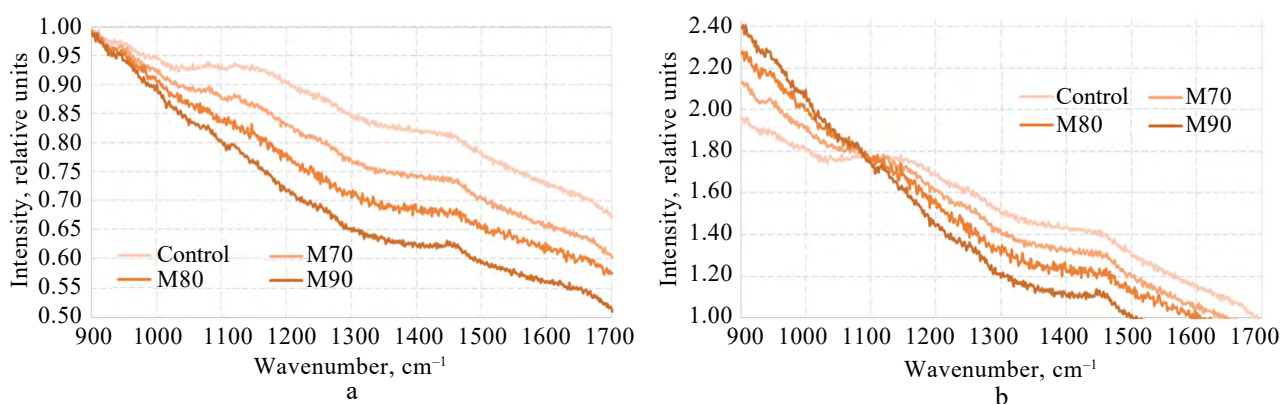
According to other studies [67, 68], the bands at  $\sim 1440 \text{ cm}^{-1}$  characterize the  $\text{CH}_2$  groups and may be used to assess heat-induced protein transformations. Similarly, Yazgan *et al.* [69] linked the spectral region of 1470–1521  $\text{cm}^{-1}$  to the deformation of methylene groups.

The previously established changes in the proteomic profile were consistent with the changes in their spectra. The complexation of  $\beta$ -lactoglobulin and  $\kappa$ -casein alters the amide bands at 1475–1546  $\text{cm}^{-1}$ , i.e., Amide II ( $\sim 1553 \text{ cm}^{-1}$ ) [68]. This region characterizes the vibrations of C-O, C-C and C-O-H bonds in the amino acid residues of aspartic and glutamic amino acids, the

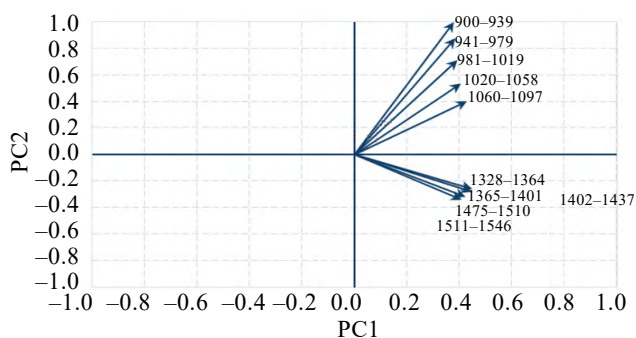
stretching of C-N bonds in peptide groups, and the deformation of N-H bond [68]. This zone reflects alterations in the microenvironment of amino acid side chains in milk proteins, caused by denaturation and aggregation of whey proteins. The observed heat-induced decrease in intensity in this region may be associated with the unfolding of protein globules or the redistribution of hydrogen bonds, as well as protein-to-protein and protein-to-carbohydrate interactions [68]. Thus, each of the identified zones of the Raman spectrum is closely associated with protein modifications during milk heating, i.e., the carbohydrate-to-protein interactions, the rearrangement of the  $\text{CH}_2$  groups in amino acid residues, the protein denaturation, and the interaction between whey proteins and casein. These spectral characteristics of pasteurized milk were consistent with the results we obtained by MALDI-TOF and two-dimensional electrophoresis. These results also demonstrated that increasing the heat load from 70 to 90°C led to alterations in the peptide profile and a reduction in whey protein concentration.

We performed a principal component analysis (PCA) of the spectral data to identify spectral regions potentially sensitive to heat load. Figures 5 and 6 visualize the heat-induced changes in the spectral profiles.

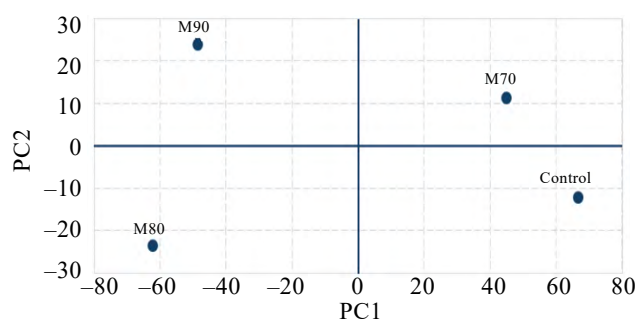
The PCA plotting visualizes the heat-induced variability of spectral profiles. The concentration of maximum loadings in a limited number of ranges indicates that the spectral changes are structurally determined and associated



**Figure 4** Raman spectra of skim milk samples: a – spectral plot after scaling (MinMaxScaler); b – spectral plot after Z-transformation (principal component analysis)



**Figure 5** Raman spectral contributions to the variability of pasteurized skim milk samples



**Figure 6** Discrimination of pasteurized skim milk samples based on their spectral characteristics: Principal component

with the transformations of certain chemical bonds in protein molecules and their functional groups. The high total variance of PC1 and PC2 indicates that the selected spectral features reflect heat-induced changes in the milk system.

According to the analysis of principal component loadings, the range of 900–939  $\text{cm}^{-1}$  had the greatest contribution to data variance: PC1 = 0.378; PC2 = 1.000; load vector magnitude = 1.070. It corresponded to the fluctuations in C-O and C-O-H lactose bonds. The high loading vector magnitude value indicated the contribution of this spectral region to the variability of milk samples across the heat-load modes. In addition, it may indicate the carbohydrate profile transformation during heat treatment and the formation of Maillard reaction products, i.e., glycated regions of protein molecules.

The next significant contribution belonged to the bands at 941–979 and 981–1019  $\text{cm}^{-1}$  with loading vector magnitudes of 0.96 and 0.81, respectively. They had positive loading values for PC1 (0.38–0.39) and PC2 (0.71–0.88). The spectral features in this region are attributed to the C-O stretching vibrations of carbohydrates. Furthermore, this spectral region may be a characteristic of protein matrix transformation detected during electrophoretic separation of milk samples. These characteristics include the redistribution of casein fraction forms and intermolecular protein interactions.

The wavenumber ranges at 1020–1058 and 1060–1097  $\text{cm}^{-1}$  (PC1 = 0.405–0.428; loading vector magnitude = 0.59–0.67) characterize the contribution of the carbohydrate fraction to the variations in pasteurized milk samples.

The spectral regions that reflect the changes in protein molecules also make a significant contribution to the dispersion of these samples across the heat-load modes: 1328–1364; 1365–1401; 1402–1437; 1475–1510; and 1511–1546  $\text{cm}^{-1}$  (loading vector magnitude = 0.52–0.53). These wavenumber regions characterize the deformation vibrations of  $\text{CH}_2$  groups in amino acid side chains and the alterations in the amide bands (Amide II), which are all sensitive to the changes in the secondary structure of protein molecules.

The results of the principal component analysis were consistent with the heat-induced changes in the milk proteomic profile previously registered by two-dimensional electrophoresis and MALDI-TOF MS: whey proteins disappeared and new protein fragments appeared as a result of intermolecular protein interactions.

Thus, the mathematical processing of spectral profiles made it possible to visualize the heat-induced changes in pasteurized skim milk. Raman spectroscopy proved to be an efficient method of assessing the effects of heat load on skim milk and the feasibility of further tests.

## CONCLUSION

In this research, molecular analysis methods were able to detect and identify heat-induced changes in skim milk proteomic profile. As the heat load increased from 70 to 90°C, two-dimensional electropherograms detected a significant decrease in the number of detectable protein frac-

tions. The control sample had 28 protein spots while the skim milk sample heated to 90°C demonstrated the lowest peptide diversity but contained new fragments associated with heat-induced proteomic transformations.

The most pronounced changes occurred in whey proteins.  $\beta$ -Lactoglobulin fractions (20, 21) and bovine serum albumin started disappearing already at 70°C. At  $\geq 80^\circ\text{C}$ , we observed a complete loss of detectable fragments of  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin, which indicated profound changes in the secondary structure and aggregation of proteins.

Although the casein fraction exhibited superior thermal stability, it nevertheless showed the formation of additional peptide fragments and a redistribution of isoforms. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) identified 26 peptides in the casein fraction. The sample treated at 90°C revealed new amino acid sequences of  $\beta$ -casein (isoform X2, Fragment 30) and  $\alpha_{s1}$ -casein (Fragment 31), which were absent from the control and the samples subjected to milder thermal treatment. The calculated and experimental isoelectric points for  $\alpha_{s1}$ - and  $\alpha_{s2}$ -casein fragments ranged from 4.75 to 8.80 pH units. This range indicated heat-induced partial dephosphorylation, glycosylation, and new covalent protein-to-protein complexes.

The method of Raman spectroscopy revealed heat-induced changes at 900–1097; 1328–1437; and 1475–1546  $\text{cm}^{-1}$  associated with carbohydrate fragments,  $\text{CH}_2$  groups of amino acid residues, and Amide II.

The principal component analysis revealed a clustering of samples based on their thermal history. The ranges characterizing the transformation of carbohydrate and protein profiles contributed most to the data dispersion: 900–939  $\text{cm}^{-1}$  (load vector modulus = 1.07); 941–979  $\text{cm}^{-1}$  (load vector modulus = 0.96); 1328–1364; 1365–1401; 1402–1437; 1475–1510; and 1511–1546  $\text{cm}^{-1}$  (load vector modulus = 0.52–0.53).

The resulting molecular and spectral changes correlated with the technological transformations in milk. On the one hand, the extensive whey protein denaturation and  $\beta$ -lactoglobulin- $\kappa$ -casein complexation adversely affect cheese-making properties and milk powder solubility. On the other hand, these changes may enable targeted modulation of curd structure in fermented dairy products.

Our study established a reliable consistency between the data obtained from two-dimensional electrophoresis, MALDI-TOF MS, and Raman spectroscopy of pasteurized milk. The heat-induced changes in the proteomic profile that were recorded during the peptide profiling found a reflection in particular spectral ranges. These correlations provide a scientifically valid platform for developing new markers of heat exposure for dairy quality control at various processing stages. These markers will establish a framework for using Raman spectroscopy to classify the thermal history of milk powder, ensuring its optimal application across different dairy production pathways.

## CONTRIBUTION

All the authors were equally involved in the manuscript and are equally responsible for any potential plagiarism.

## CONFLICT OF INTEREST

The authors declared no potential conflict of interest regarding the research, authorship, and / or publication of this article.

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