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# DISTRIBUTION OF CASEIN MOLAR FRACTIONS IN PASTA FILATA CHEESES

## DISTRIBUCE MOLÁRNÍCH FRAKCÍ KASEINU U PAŘENÝCH SÝRŮ

DOCTORAL THESIS

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**ABSTRACT**

This research is focused on the study of changes in the casein complex in *pasta filata cheeses*. The aim of the study was to analyze the degradation of casein complex in various types of Czech pasta filata cheeses. To achieve this objective the basic chemical parameters of cheese curds and final products were analyzed. The basic technological parameters i.e. actual acidity values (pH), titratable acidity values (SH°) and the influence of different temperatures and time of heating for the cheese curds mass were studied. These factors directly affect the quality of heating process and the final cheese product.

The experimental samples of cheeses were collected from four dairy industrial organizations. The experiments were based on analysis of cheese curd before heating, after heating and following analysis of final products during ripening or, at the end of expiration date. The casein complex degradation range and depth was monitored by Gel Permeation Chromatography (GPC) and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The obtained values were evaluated on standard samples of acid-precipitated casein (fraction of α-, β-, and κ-caseins). As additional analyses a microbiological analysis, analysis of biogenic amines and sensory evaluation were carried out.

The analyses results show that casein complex is relatively thermostable, i.e. under steaming standard temperature used for this technology the denaturation and degradation of the casein complex was within the tolerance. The GPC method as well as SDS-PAGE are applicable and meaningful for studies on changes in the casein complex.

Key words: pasta filata cheese, casein, pH, SDS-PAGE, GPC

**ABSTRAKT**

Tato práce je zaměřena na studium změn kaseinového komplexu u pařených sýrů typu *Pasta Filata*. Hlavním cílem bylo sledování degradace kaseinového komplexu u různých druhů vyrobených pařených sýrů s respektováním jejich odlišnosti a s ohledem na charakter zrání a druh pařeného sýra. Pro dosažení tohoto cíle byly sledovány základní chemické parametry suroviny (sýrového těsta) pro výrobu pařených sýrů. U standardně vyrobené suroviny, která je základem pro výrobu pařených sýrů byly ověřeny rozhodující technologické parametry tj. hodnoty prokysáni suroviny (pH), vliv rozdílné teploty a doby paření suroviny. Uvedené faktory bezprostředně ovlivňují proces kvality paření sýrů a finálního výrobku.

Experimenty byly založeny na analýze suroviny před pařením, po paření a následně analýzy finálních produktů v průběhu zrání resp. na konci doby min. trvanlivosti.

Rozsah a hloubka degradace kaseinového komplexu byla sledována pomocí gelové permeační chromatografie (GPC) a metodou sodium dodecylsulfat polyakrylamidovou gelovou elektroforezou (SDS–PAGE). Zjištěné hodnoty byly porovnány na standardní vzorek kysele sráženého vzorku kaseinu (frakce α-, β-, κ –kaseinů ). Jako doplňující analýzy byl provedeny mikrobiální rozbory, rozbory na biogenní aminy a senzorická analýza.

Z výsledků analýz vyplývá, že kaseinový komplex je poměrně termostabilní, tzn. že standardní pařicí teploty používané pro tuto technologie vyvolávají jen v omezeném rozsahu denaturaci a degradaci kaseinového komplexu. Jak metoda GPC, rovněž tak SDS-PAGE jsou použitelné a vypovídající o změnách kaseinového komplexu. Následná hlubší proteolýza kaseinového komplexu je pak vyvolána proteolytickými enzymy produkovaným mikroorganismy.

Klíčová slova: pařený sýr, kasein , pH, SDS–PAGE, GPC

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## LIST OF ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| GPC | Gel Permeation Chromatography |
| SDS-PAGE | Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis |
| β–Lg | β–lactoglobulin |
| α–La | α–lactalbumin |
| CCC | Colloidal calcium phosphate |
| LAB | Lactic acid bacteria |
| NSLAB | Non-starter lactic acid bacteria |
| BAs | Biogenic amines |
| pH | Actual acidity |
| SH° | Soxhlet–Henkel degree |
| DRI | Differential refractometer |
| VIS | Viscometer detectors |
| MWD | Molecular weight distribution |
| $M_w$ | Weight average molecular weight |
| $M_n$ | Number average molecular weight |
| PDI | Polydispersity index |
| SD | Standard deviation |
| m.w. | Molecular weight |
| kDa | Kilodalton |
| ND | Not detected |

# 1. LITERATURE REVIEW

## 1.1 Overview of milk proteins

Milk is a very complex fluid containing several hundred molecular species (several thousand if all triglycerides are counted individually). The properties of milk and most dairy products are affected more by the proteins they contain than by any other constituent. The milk proteins have been studied extensively and still under discussion [1].

### 1.1.1 Characterization and properties of milk proteins

Cow's milk is mainly composed of about 87% water [1, 2], approximately 4.8% lactose, 3.2% protein, 3.7% fat, 0.19% non-protein nitrogen and 0.7% ash [1, 2, 3]. Technologically, the milk proteins are probably the most important constituents of the milk, due to their unique properties [4]. They play important, even essential, roles in all dairy products. The roles played by milk proteins include:

- ✓ Nutritional: all protein-containing dairy products.
- ✓ Physiological: immunoglobulins, lactoferrin, lactoperoxidase, vitaminbinding proteins, protein-derived biologically active peptides.
- ✓ Functional:

    - gelation: enzymatically, acid or thermally induced gelation in all cheeses fermented milks, whey protein concentrates and isolates;
    - heat stability: all thermally processed dairy products;
    - surface activity: caseinates, whey protein concentrates and isolates;
    - rheological: all protein-containing dairy products;
    - water sorption: most dairy products and in food products containing functional milk proteins .

It has been known since 1830 that milk contains two types of protein which can be separated by acidification to pH 4.6 [5]. The most versatile structural element involved in technological transformations is the protein conglomerate called *casein*. The casein insoluble at pH 4.6, is in the form of colloidally dispersed particles, known as micelles and represent ~80% of the total nitrogen

in bovine [1, 5, 6, 7], buffalo [8], caprine [9] milk; the soluble proteins are called *whey* or *serum* proteins and represent ~20 % [1, 3, 4, 9]. The pioneering work in this area was done by the German scientist, Hammarsten, and consequently isoelectric (acid) casein is sometimes referred to as *casein nach Hammarsten* [10]. Now known that the two principal whey proteins, milk–specific–β–lactoglobulin (β–Lg) and α–lactalbumin (α–La) [4, 11].

The concentration of total protein in milk is affected by most of the same factors that affect the concentration of fat, i.e., breed, individuality, nutritional status, health and stage of lactation, but with the exception of the last, the magnitude of the effect is less than for milk fat. The concentration of protein in milk decreases very markedly during the first few days post-partum [5, 11], mainly due to the decrease in Ig (immunoglobulin) from ~10% in the first colostrum to 0.1% within about one week. The concentration of total protein continues to decline more slowly thereafter to a minimum after about four weeks and then increases until the end of lactation [5].

Milk contains six milk-specific proteins: four caseins, $\alpha_{s1}$–, $\alpha_{s2}$–, β– and κ–, representing approximately 38%, 10%, 35% and 15%, respectively, of whole casein [5, 12, 13, 14], and β-Lg and α-La ,which represent approximately 40% and 20%, respectively, of total whey proteins [5]. Table 1 shows content of major protein components in milk, with different genetic variants of each caseins, and several minor proteins originating from postsecretion proteolysis of the primary caseins.

The application of electrophoresis in starch or polyacrylamide gels, which were introduced about 1960, showed that the milk protein system is very heterogeneous [4], by the fact that the caseins are products of co-dominant allelic autosomal genes [16] due to:

- ✓ genetic polymorphism, usually involving substitution of one or two amino acids;
- ✓ variations in the degree of phosphorylation of the caseins;
- ✓ variations in the degree of glycosylation of κ–casein;
- ✓ intermolecular disulphide bond formation in $\alpha_{s1}$– and κ–caseins
- ✓ limited proteolysis by plasmin, especially of β– and $\alpha_{s1}$–caseins; the resulting peptides include the γ– and λ–caseins and proteose peptones [5].

**Table 1.** Content of the major protein components in milk [15].

| | Content of Protein in Milk | |
|---|---|---|
| Protein Type | Protein or Polypeptide | Weight Contribution (g/L) |
| Casein | —— | 24-28 |
| | $\alpha_{s1}$-Casein | 12-15 |
| | $\alpha_{s2}$-Casein | 3-4 |
| | β-Casein | 9-11 |
| | κ-Casein | 3-4 |
| | γ-Casein | 1-2 |
| Whey protein | —— | 5-7 |
| | β-Lactoglobulin | 2-4 |
| | α-Lactalbumin | 1-1.5 |
| | Bovine serum albumin | 0.1-0.4 |
| | Immunoglobulins | 0.6-1.0 |
| | Proteoses peptones | 0.6-1.8 |

The properties of principal caseins and their genetic variants are summarized in Table 2.

**Table 2.** Properties of the principal caseins in cows' milk [6, 17].

| | **Molecular mass (Da)** | **AA residues** | | | **$PO_4$** | **Genetic variants** |
|---|---|---|---|---|---|---|
| | | Total | Pro | Cys | | |
| $\alpha_{s1}$ - Casein | 23 614 | 199 | 17 | 0 | 8 | A,B,C,D,E,F,G,H |
| $\alpha_{s2}$ - Casein | 25 388 | 207 | 10 | 2 | 10-13 | A,B,C,D |
| β - Casein | 23 983 | 209 | 35 | 0 | 5 | $A^1$,$A^2$,$A^3$,B,C,D,E,F,G |
| κ - Casein[a] | 19 038 | 169 | 20 | 2 | 1 | A,B,C,E,$F^s$,$F^I$,$G^S$,$G^S$,H,I,J |
| β- lactoglobulin | 18 277 | 162 | 8 | 5 | 0 | A,B,C,D,E,F,H,I,J |
| α- lactoalbumin | 14 175 | 123 | 2 | 8 | 0 | A,B,C |

Da-Dalton, a-Glycosylated to variable extent

The principal proteins are very well characterized at the molecular level. The most notable features of the principal milk-specific proteins are as follows:

- All milk proteins are quite small molecules, ~15–25 kDa;

- All the caseins are phosphorylated but to different and variable degrees; the phosphate groups are esterified as monoesters of serine residues;

- $\alpha_{s1}$-casein and $\alpha_{s2}$-casein are calcium sensitive and can be precipitated at very low levels of calcium;

- κ-casein is positioned on the outside of the casein micelle. Unlike the other caseins κ-casein is very resistant to calcium precipitation, stabilizing other caseins. Rennet cleavage at the Phe105-Met106 bond eliminates the stabilizing ability, leaving a hydrophobic portion, para–κ–casein, and a hydrophilic portion called κ–casein glycomacropeptide, or caseinomacropeptide. Cleavage of this bond is the first step in the coagulation of milk by aggregation of the casein micelles after the loss of the hydrophilic, negatively charged surface from the micelle [15,16];

- β–casein is the only one of the principal milk proteins that is glycosylated. β–casein is a very amphiphilic protein, and that's why it acts like a detergent molecule. The protein's self-association depends on temperature. It will form a large polymer at 20° C, but not at 4° C. This type of casein is less sensitive to calcium precipitation [16];

- All the caseins, especially β–casein, contain a high level of proline, which disrupts α– and β– structures; consequently, the caseins are rather unstructured molecules and are readily susceptible to proteolysis. However, theoretical calculations suggest that the caseins may have a considerable level of secondary and tertiary structures; to explain the differences between the experimental and theoretical indices of higher structures, it has been suggested that the caseins have very mobile, flexible structures and are referred to as rheomorphic [5, 16].

- The two principal caseins, $\alpha_{s1}$– and β–, are devoid of cysteine or cystine residues; the two minor caseins, $\alpha_{s2}$– and κ–caseins, contain two intermolecular disulphides. β–Lg contains two intramolecular disulphides and one sulphydryl group which is buried and unreactive in the native protein but becomes exposed and reactive when the molecule is denatured; it reacts via sulphydryl–disulphide interactions with other

proteins [17], especially κ-casein, with major consequences on many important properties of the milk protein system, especially heat stability and cheesemaking properties. α-La has four intramolecular disulphides;

- In contrast, the whey proteins are highly structured and compact, with high levels of α–helices, β–sheets and β–turns. In β–Lg, the β–sheets are in an antiparallel arrangement and form a β–barrel calyx [5, 18];

- The caseins are often regarded as rather hydrophobic proteins but they are not particularly so; however, they do have a high surface hydrophobicity owing to their open structure; in globular proteins, the hydrophobic residues are buried within the molecule but they are exposed in the caseins;

- Also due to their open structure, the caseins are quite susceptible to proteolysis, which accords with their putative function as a source of amino acids for the neonate. However, their hydrophobic patches give them a high propensity to yield bitter hydrolysates, even in cheese which undergoes relatively little proteolysis [5];

- Probably because of their rather open structures, the caseins are extremely heat stable, e.g., sodium caseinate can be heated at 140ºC for 1 h without obvious physical effects. The more highly structured whey proteins are comparatively heat labile, although in comparison with many other globular proteins, they are quite heat stable; they are completely denatured on heating at 90ºC for 10 min;

- Under the ionic conditions in milk, α–La exists as monomers of MW ~14.7 kDa. β–Lg exists as dimers (MW ~ 36 kDa) in the pH range 5.5–7.5; at pH values <3.5 or >7.5 it exists as monomers, while at pH 3.5–5.5 it exists as octamers [13,14];

- The function of the caseins appears to be to supply amino acids to the neonate. They have no biological function *sensu stricto* but their Ca-binding properties enable a high concentration of calcium phosphate to be carried in milk in a ‘soluble’ form; without the ‘solubilizing’ influence of casein, $Ca_3(PO_4)_2$ would precipitate in the ducts of the mammary gland and cause atopic milk stones;

- β–Lg binds several hydrophobic molecules; it binds and protects retinol *in vitro* and perhaps functions as a retinol carrier *in vivo*. In the intestine, it may exchange retinol with a retinol–binding protein. It also binds fatty acids and thereby stimulates lipase – perhaps this is its principal biological function. All members of the lipocalin family have some form of binding fiction [17,21].

- α–La is a metalloprotein – it binds one calcium atom per molecule in a peptide loop containing four Asp residues. The apoprotein is quite heat labile but the metalloprotein is rather heat stable; the difference in heat stability between the halo– and apoprotein is exploited in the isolation of α–La on a potentially industrial scale[17,22];

- α–La is a specifier protein in lactose synthesis; it makes UDP–galactose transferase highly specific for glucose as an acceptor of galactose, resulting in the synthesis of lactose [4, 10, 11, 14, 15, 18-22].

### 1.1.2 Casein micelle

Due to the importance of casein and casein micelles for the functional behavior of dairy products, the nature and structure of casein micelles have been studied extensively [23-31].

In normal milk, about 95% of the casein proteins exist as coarse colloidal particles, called micelles, with diameters ranging from 80 to 300 nm (average ~150 nm). These particles are formed within the secretory cells of the mammary gland and undergo relatively little change after secretion. On a dry weight basis, the micelles consist of ~94% protein and ~6% of small ions, principally calcium, phosphate, magnesium and citrate, referred to collectively as colloidal calcium phosphate (CCP). The κ-casein content of casein micelles is inversely proportional to their size, while the content of CCP is directly related to size [31, 32,33].

The micelles are very open and highly hydrated structures containing about 2–4 g $H_2O$ per g protein, depending on the method of measurement. The apparent zeta potential for casein micelles is about –19 mV at 25°C. The structure of the micelle is dynamic, e.g., cooling the milk to about 4°C causes

solubilization of a significant proportion of β–casein and some κ–casein and much lower levels of $\alpha_{S1}$–and $\alpha_{S2}$–caseins [32].

The precise structure of the casein micelle is a matter of considerable debate at the present time. A number of models have been proposed over the past 40 years, but none of them can describe completely all aspects of casein micelle behavior [23].Most of the proposed models fall into three general categories, which are: coat–core, subunit (sub–micelles), and internal structure models. The coat–core models, proposed by Waugh and Nobel in 1965, Payens in 1966, Parry and Carroll in 1969, and Paquin and co-workers in 1987, describe the micelle as an aggregate of caseins with outer layer differing in composition form the interior, and the structure of the inner part is not accurately identified. The sub–micelle models, proposed by Morr in 1967, Slattery and Evard in 1973, Schmidt in 1980, Walstra in 1984, and Ono and Obata in 1989, is considered to be composed of roughly spherical uniform subunits. The last models, the internal structure models, which were proposed by Rose in 1969, Garnier and Ribadeau – Dumas in 1970, Holt in 1992 [32], and Horne in 1998, specify the mode of aggregation of the different caseins [23].

In the sub–unit models, caseins are aggregated to form sub-micelles (10–15 nm in diameter). It has been suggested that sub–micelles have a hydrophobic core that is covered by a hydrophilic coat. The polar moieties of κ–casein molecules are concentrated in one area. The remaining part of the coat consists of the polar parts of other caseins, notably segments containing their phosphoserine residues. The sub–micelles are assumed to aggregate into micelles by CCP which would bind to $\alpha_{S1}$–, $\alpha_{S2}$– and β–caseins via their phosphoserine residues. Submicelles with no or low κ–casein are located in the interior of the micelle whereas κ–casein rich sub–micelles are concentrated on the surface [23, 30, 32].

Other models consider the micelle as a porous network of proteins (of no fixed conformation); the calcium phosphate nanoclusters are responsible for crosslinking the protein and holding the network together. More specifically there are no subunits because individual polypeptide chains with two or more phosphate centers provide a network of strong interactions that link together most of the Ca–sensitive caseins in a micelle. The surface layer is a natural extension of the internal structure. A recent model proposed by Horne [34] assumes that the assembly of the casein micelle is governed by a balance of

electrostatic and hydrophobic interactions between casein molecules. As stated earlier, $\alpha_{S1}$–, $\alpha_{S2}$– and β–caseins consist of distinct hydrophobic and hydrophilic regions. Two or more hydrophobic regions from different molecules form a bonded cluster. Growth of these polymers is inhibited by the protein charge residues, the repulsion of which pushes up the interaction free energy. Neutralization of the phosphoserine clusters by incorporation into the CCP diminishes that free energy as well as producing the second type of cross-linking bridge. κ–casein acts a terminator for both types of growth, as it contains no phosphoserine cluster or another hydrophobic anchor point[30, 32].

A common factor in all models is that most of the κ-casein appears to be present on the surface of casein micelles. The hydrophilic, C–terminal part of κ–casein, is assumed to protrude 5 to 10 nm from the micelle surface into the surrounding solvent, giving it a "hairy" appearance and providing a steric stabilizing layer. The highly charged flexible "hairs" physically prevent the approach and interactions of hydrophobic regions of the casein molecules [24, 30, 32].

Despite a wide variety of genetic influences that can alter the ratios of the individual caseins, casein micelles are formed in a biologically competent fashion to allow the secretion of the completed micelles. The combination of past research on the details of this biological process and recent developments from the studies of protein-protein interactions in the field of protein science leads to the following conclusions:

- Selective and productive proteinprotein interactions (electrostatic and hydrophobic, etc.) are the driving force in the formation of casein micelles;
- On transport to the Golgi apparatus, the pre-formed proteinaceous particles (submicelles) are phosphorylated (rather slowly) and calcium and phosphate intercalated into these particles;
- Casein association/aggregation occurs in the Golgi vesicles through the coupling of the "submicelles" like (~20 nm) complexed with calcium and phosphate-casein micelles;
- It is likely that casein micelle formation is a hierarchal process-originated from a basic protein-protein interaction unit (~ 9.0–11 nm), which may or may not lead to the successful formation of micelles [23].

### 1.1.3 Casein micelle stability

The micelles are stabilized by two principal factors: (1) a surface potential of C 20 mV at pH 6.7 which alone is insufficient for colloidal stability and (2) steric stabilization due to protruding κ–casein hairs. Casein micelles can be caused to aggregate by several factors. Much attention has been focused on the curd formation during cheese making brought about by the action of chymosin which destroys the stabilizing effect of κ–casein. Chymosin is highly specific in its action, splitting the κ–casein at the Phe105–Met106 bond, releasing the hydrophilic peptide and destabilising the micelles. This action results in a decrease in the micellar zeta potential from about –20 to –10 mV, and prior to aggregation a decrease in micellar hydrodynamic size as the hairy layer is cleaved off. Many other proteases with a more general action can also hydrolyze a specific bond of κ–casein, resulting in micelle aggregation [20, 30, 32, 34, 35].

Reducing the pH of milk has several significant implications for the physicochemical properties of the casein micelles (Fig.1), and hence the properties of milk. Casein micelles aggregate and precipitate from solution when the pH is lowered to about pH 4.6. When the pH of milk is reduced, CCP is dissolved and the caseins are dissociated into the milk serum phase [32]. The extent of dissociation of caseins (especially β–casein) is dependent on temperature of acidification; at 30°C, a decrease in pH causes virtually no dissociation; at 4°C about 40% of the caseins are dissociated in the serum at pH ~5.5. Aggregation of casein occurs as the isoelectric point (pH 4.6) is approached. Apparently little change in the average hydrodynamic diameter of casein micelles occurs during acidification of milk to pH~5.0. The lack of change in the size of micelles on reducing the pH of milk to 5.5 may be due to concomitant swelling of the particles as CCP is solubilized. The mobility of casein micelles measured by nuclear magnetic resonance spectroscopy does not change with pH [34].

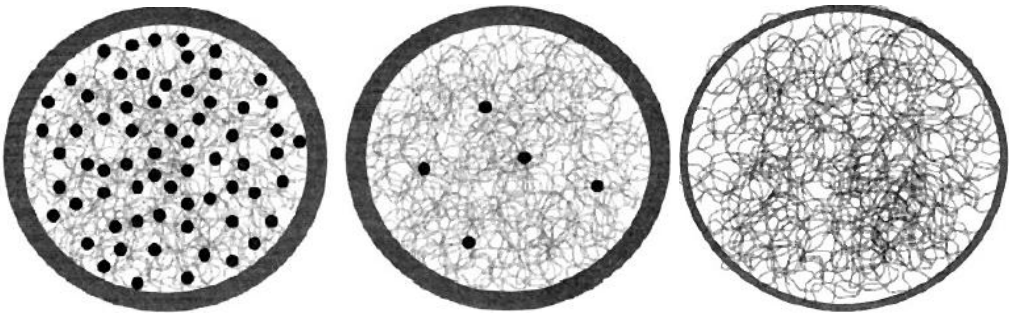


**Figure 1**: Representations of casein micelle structures at various pH values as indicated [20].

Casein micelles are very stable at high temperatures, but they can aggregate and coagulate after heating at 140°C for 15–20 min. Such coagulation results from a number of changes in milk systems that occur during heating, including a decrease in pH, denaturation of whey proteins and their association with κ–casein, transfer of
soluble calcium and phosphate into colloidal state, dephosphorylation of caseins and a decrease in hydration. Upon cooling, they become more resistant to flocculation, probably owing to steric repulsion by protrusion of polypeptide chains [13, 34].

The micelles are also destabilized by addition of about 40% ethanol at pH 6.7 and by lower concentrations if the pH is reduced. This is due to the collapse and folding of the hairy layer of κ–casein in the non–solvent mixture, allowing micelles to interact and aggregate. Freezing of milk has been shown to cause destabilization of casein micelles which is due to a decrease in pH and an increase in the $Ca^{2+}$concentration in the unfrozen phase of milk [32].

### 1.1.4 Protein structure

Proteins are macromolecules with different levels of structural organization [36]. Protein structure is stabilized by multiple weak interactions. Hydrophobic interactions are the major contributors to stabilizing the globular form of most soluble proteins; hydrogen bonds and ionic interactions are optimized in the specific structures that are thermodynamically most stable [36, 37].

A description of all covalent bonds (mainly peptide bonds and disulfide bonds) linking amino acid residues in a polypeptide chain is its **primary structure.** Amino acids are small molecules that contain an amino group ($NH_2$), a carboxyl group (COOH), a hydrogen atom (H) and a side chain (R–group) attached to a central alpha carbon ($C_\alpha$). The nature of the covalent bonds in the polypeptide backbone places constraints on structure. The peptide bond has a partial doublebond character that keeps the entire six–atom peptide group in a rigid planar configuration. The N–$C_\alpha$ and $C_\alpha$-C bonds can rotate to assume bond angles of $\varphi$ and $\psi$, respectively. The most important element of primary structure is the *sequence* of amino acid residues [36, 37, 38].

Some proteolytic enzymes have quite specific actions; they attack only a limited number of bonds, involving only particular amino acid residues in a particular sequence. This may lead to the accumulation of well–defined peptides during some enzymic proteolytic reactions in foods [36].

**Secondary structure** refers to particularly stable arrangements of amino acid residues giving rise to recurring structural patterns [36]. Hydrogen bonds between amide nitrogen and carbony1 oxygen are the major stabilizing force. In aqueous media, the hydrogen bonds may be less significant, and van der Waals forces and hydrophobic interaction between apolar side chains may contribute to the stability of the secondary structure [36]. The most common secondary structures are the α-helix, the $\beta$– conformation, and $\beta$– turns. The secondary structure of a polypeptide segment can be completely defined if the $\psi$ and $\varphi$ angles are known for all amino acid residues in that segment [36, 38, 39].

The **tertiary structure** of proteins involves a pattern of folding of the chains into a compact unit that is stabilized by hydrogen bonds, van der Waals forces, disulfide bridges, and hydrophobic interactions. The tertiary structure results in the formation of a tightly packed unit with most of the polar amino acid residues located on the outside and hydrated [36]. The tertiary structure describes all aspects of the three–dimensional folding of a polypeptide chain. There are two general classes of proteins based on tertiary structure: fibrous and globular. The nature of the tertiary structure varies among proteins as does the ratio of α–helix and random coil. Insulin is loosely folded, and its tertiary structure is stabilized by disulfide bridges. Lysozyme and glycinin have disulfide bridges but are compactly folded [36, 37, 38, 39].

When a protein has two or more polypeptide subunits, their arrangement in space is referred to as **quaternary structure.** Quaternary structure results from interactions between the subunits of multisubunit (multimeric) proteins or large protein assemblies. Some multimeric proteins have a repeated unit consisting of a single subunit or a group of subunits referred to as a protomer. Protomers are usually related by rotational or helical symmetry. These structures may be stabilized by hydrogen bonds, disulfide bridges, and hydrophobic interactions [36, 37, 38].

The well-defined secondary, tertiary, and quaternary structures are thought to arise directly from the primary structure. This means that a given combination of amino acids will automatically assume the type of structure that is most stable [36, 40].

## 1.2 PASTA FILATA CHEESES

According to the Decree No. 77/2003 Coll. Ministry of Agriculture of the Czech Republic, cheese - milk product manufactured by precipitation of milk protein, action of rennet or other suitable coagulation agents, and the souring and separation of whey fractions [41].

"Formaggio a pasta filata" is the Italian name for types of cheese which in English are called *pasta filata cheese*, characterized by an "elastic" string curd [42]. The first pasta filata cheese dates back thousands of years, possibly introduced as early as the $6^{th}$ Century [42], and originated primarily in the greater northern Mediterranean region, encompassing Italy, Greece, the Balkans, Turkey and eastern Europe. The category of pasta filata cheeses includes different dairy types that differ one from another because of the raw material, technology, size, etc. Some are soft or semi-soft cheeses that are, typically, consumed fresh or after only a brief period of ageing (e.g., fresh Mozzarella, low-moisture Mozzarella, Scamorza). Others are hard or semi-hard ripened cheeses that may undergo considerable ageing before being consumed (e.g., Caciocavallo, Kashkaval, Provolone, Ragusano) [43]. Typical composition values for some of the pasta filata cheeses are presented in Table 3. Widely distributed types of pasta filata cheeses in other country: Kasseri, Kefalotiri in Greece; Cascaval in Romania; Kaškaval in Bulgaria; Slovenská parenica, Oštiepok, Liptov in Slovakia; Jadel, Polianka, Koliba in Czech Republic[44].

**Table 3.** Typical composition of some pasta filata cheeses [45].

| Cheese | Moisture (%) | Fat (%) | Total protein (%) | NaCl |
|---|---|---|---|---|
| Caciocavallo | 30 | 27 | 33 | 3.9 |
| Kashkaval | 38 | 32 | 21 | 3.0 |
| Provolone | 38 | 28.5 | 24 | 3.2 |
| Ragusano | 38 | 30 | 30 | 2.5 |
| Mozzarella, high moisture | 54 | 18 | 22 | 0.7 |
| Mozzarella, low moisture | 47 | 24 | 21 | 1.5 |
| Scamorza | 45 | 25 | 24 | 1.5 |

The pasta filata cheeses share a unique processing step towards the end of manufacture, when the curd is immersed in hot water or salt brine and mechanically worked (stretched) to a semi–flowable plastic consistency which can be formed or moulded into a variety of shapes. The process of stretching represents a significant heat treatment of the fresh curd; therefore, the practice probably originated as a means of preservation, to improve the quality and prolong the shelf-life of the cheese. In addition to causing thermal inactivation of some microorganisms and enzymes, stretching results in a striking rearrangement of curd structure that gives rise to unique textural and melting characteristics. These unique functional properties have proven to be of fundamental importance to the worldwide meteoric rise in the popularity of pasta filata cheese as a pizza ingredient [45].

By far the most important member of pasta filata cheese group is Mozzarella, and was originally manufactured from buffalo milk. Mozzarella di bufala is hand-molded into round pieces (100-300 g) during manufacture. This cheese is still manufactured in Italy, but the type of Mozzarella now widely manufactured around the world is made from pasteurized, partly skimmed cow milk [46]. The development of fast-food and franchise chain pizza restaurants further hastened the growth of pizza, which eventually became an omnipresent element of American culture. Along with the growth of pizza came an unprecedented increase in the demand for Mozzarella cheese, as evidenced by striking increases in United States production of Mozzarella over the past 25 years. More recently, American franchise restaurant chains have expanded

aggressively in other countries, thereby increasing the popularity of pizza in Europe, South America and Asia [45].

### 1.2.1 Cheese production

The basic manufacturing technology for pasta filata cheeses is not unlike that of many varieties. The pasta filata cheese making process comprises several steps (Fig. 2). Cheese manufacture commences with the selection of milk of high microbiological and chemical quality [47]. The manufacturing process for Mozzarella for use as pizza topping involves standardizing pasteurized cow milk to around 1.8% fat. A higher fat content (~ 3.6%) is used for Mozzarella intended to be consumed as a table cheese. Starter culture used in cheese manufacture can be classified into two large groups: traditional (including artisanal, natural starters and mixed-strain starters) and defined starters [48]. The principal role of the starter culture is to produce enough lactic acid during cheesemaking to demineralize and transform the curd into a state that will stretch in hot water at the target pH. Furthermore, acidification must proceed at a rate that will allow for adequate syneresis during cheesemaking to achieve the target moisture content. The starter culture can be eliminated altogether and replaced by direct acidification of the cheesemilk in the manufacture of traditional Mozzarella or low–moisture Mozzarella (pizza cheese), provided that the appropriate level of demineralization in combination with an appropriate pH at stretching are achieved. A secondary role of the starter in aged pasta filata cheeses, including pizza cheese, is secondary proteolysis. However, the extent and significance of starter associated proteolysis varies widely depending on the cooking and stretching temperatures used and the extent of thermal inactivation of the coagulant enzymes and starter culture organisms that results [45].

A thermophilic starter (1-2%) containing a combination of *Lactobacillus spp*. and *Streptococcus Thermophilus* is used in the manufacture of pizza cheese. The *Lactobacillus* is often omitted when Mozzarella is intended as a table cheese, since the rate of acidification need not be as fast as in pizza cheese. Proteolytic enzymes of the *Lactobacillus* may make a minor contribution to the functionality of the final product by causing slight hydrolysis of the caseins. Mozzarella cheese made using direct acidification generally has a softer body and better melting quality than cheese of similar age made with starter culture.

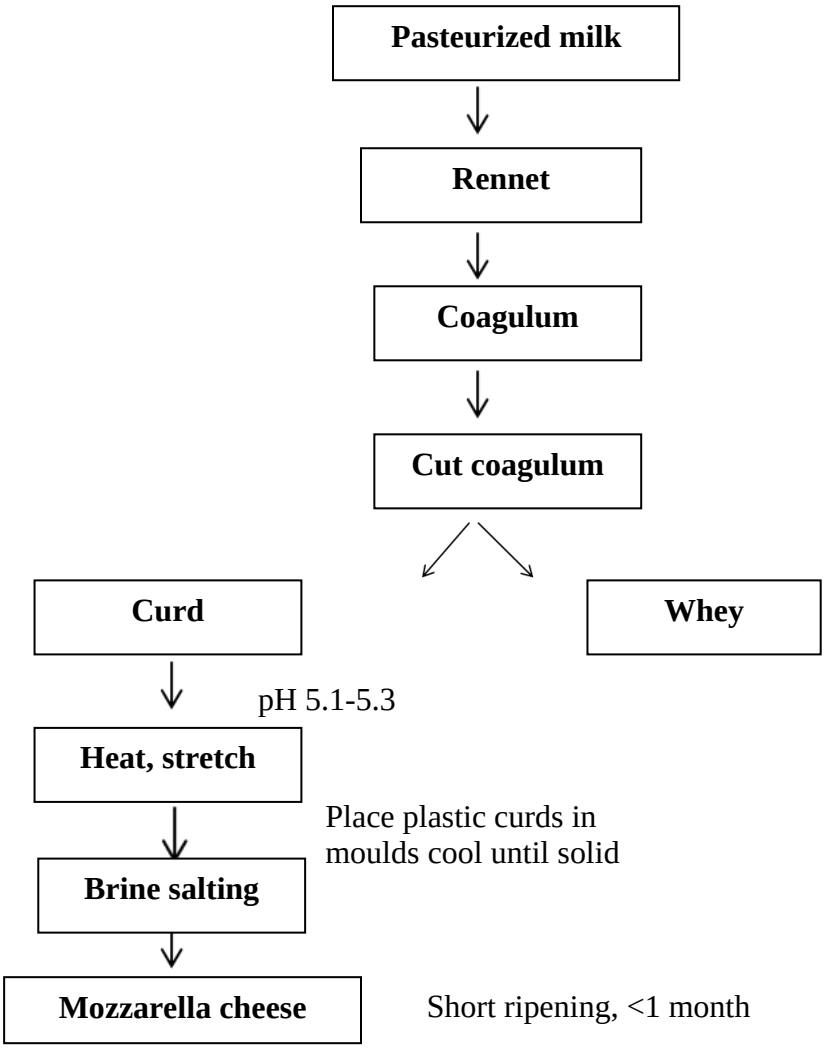


**Figure 2**. Overview of the cheese making process [46].

The milk is renneted after some acidity has developed, and the coagulum is cut and cooked to around 41 °C. The whey is then usually drained off, and texture is developed in the curds (usually by cheddaring) until the pH drops to around pH 5.1-5.3. [46], this gives the cheese a characteristic fibrous structure [49].

The next stages in Mozzarella manufacture are stretching and kneading, which are characteristic of pasta filata varieties (Figure 3). The curds are placed in hot water (~ 7O °C) and kneaded, stretched, and folded until the desired texture has been developed. The curds for pizza cheese are stretched more extensively than those for table Mozzarella. The former may also be salted during the stretching and forming stages.

**Figure 3**. Hot homogeneous mass of cheese as it exits the cooker/stretcher [50].

The hot, plastic curds are molded (usually into rectangular blocks) and cooled quickly in cold water or brine, and if salt was not added during the cooking and stretching process, the cheeses are then brine-salted [46, 51, 52]. Alternatively, immerse the cold, shaped cheese in 16-2 % salt brine at 8-10 °C for sufficient time (5 min to 24 h) to allow 1.6% of salt in the cheese. Brine strength and the size of the cheese dictate the time in brine. Dry off the cheese for an hour after salting in brine [53], may contain 45-60% moisture [54]. Mozzarella is usually consumed within a few weeks of manufacture. Extensive ripening is undesirable, since the functional properties of the cheese deteriorate [46].

### 1.2.2 Effect of acidity raw material (pH), calcium, temperature on formation of cheese structure

Mozzarella cheese is a very complex material and its properties are affected by many factors, among which state of water (bound, entrapped or bulk), the state of fat (globular or pools trapped within voids in the protein matrix), the extent of protein association (through calcium phosphate bonds or

hydrophobic interaction), the pH, and the mineral and ionic balance (especially sodium chloride and calcium) [55, 56].

The pH of cheese curd is one of the distinguishing characteristics, that influences rates of enzymatic and bacterial activity of the curd and major determinants of microstructure in Mozzarella cheese [57]. Mozzarella cheese curd is normally cooked at 40 °C or higher, which removes moisture from cheese and causes some inactivation of chymosin and starter culture microorganism. Higher cooking temperature lowers cheese moisture content and rate of proteolysis, and hence lowers cheese meltability and stretchability [58]. When cook temperature is reduced to 35°C, the curd retains more moisture, which results in a softer cheese and a higher level of proteolysis after the cheese is made. The breakdown of $\alpha_{s1}$–casein that takes place during extended storage weakens the cheese further and eliminates textural and melting problems often experienced with reduced-fat Mozzarella. At a pH of 5.2 to 5.4, di-calcium paracaseinate is converted into mono-calcium paracaseinate by the action of lactic acid and imparts cheese a stringy texture and sheen. At a pH greater than 5.4, curd will not stretch; at a pH less than 5.2, excessive fat losses occur, and the cheese becomes too tough. Curd stretched at pH 5.3 has a more structured texture and takes longer to age. Curd stretched at pH 5.3 exhibited higher apparent viscosity immediately after manufacture and during aging compared to Mozzarella cheese made from curd stretched at pH 5.0. For optimal stretching, there is an optimal combination of curd pH and stretching temperature. Scott *et al*. [53] indicated that curd at pH 5.1–5.4 should be placed in hot water at 70 to 82°C for stretching. Mulvaney *et al.* [59] reported a reduction in elastic properties of Mozzarella when the stretching temperature of the curd was increased from 57 to 75°C.

Another effect of higher stretching temperature is increased inactivation of proteolytic organisms and residual enzymes and a concomitant reduction in primary and secondary proteolysis during aging. Apostolopoulos *et al*. [60] compared Mozzarella cheese made with a conventional cooker/stretcher to that made using a high-pressure, twin-screw extruder. The extruder stretching resulted in a cheese with lower meltability and no detectable free oil. Stretched curd is cooled in chilled water-cooling towers or by other means while the Mozzarella cheese is still in molds. This is performed at a high rate to limit growth of certain undesirable microorganisms, such as *L. caseii*, which may lead to soft-body texture defect and gas holes. Soft-body defect renders cheese soft

and pasty with poor shredding qualities and excessive meltability. Cooling continues to occur when Mozzarella cheese is placed in brine for salting. At this stage, a nonuniform salt and moisture gradient is established in the cheese block [61] and eventually leads to variations in cheese meltability, stretchability, free-oil formation, etc., at different locations within the block [62].

The pH dictates the amount of calcium that is partitioned into the curd structure at the point of draining of the whey, and also the ratio of soluble to insoluble calcium in the final cheese. Insoluble calcium, which is bound to protein in cheese directly, contributes to cheese protein microstructure, as the protein fibres are more closely associated through calcium phosphate bridging [63, 64, 65]. At lower pH the proportion of ionic soluble calcium rises which will assist in shielding the charges on the proteins, thus allowing association of the proteins through hydrophobic interaction. These two types of interaction are strongly pH–dependent and produce different types of cheese texture [56]. Yun *et al.* [58] investigated the effects of pH at milling on the composition and functional properties of Mozzarella cheese. Milling cheese curd at pH 5.10, 5.25, or 5.40 did not affect meltability or textural properties of cheese, but the apparent viscosity of melted cheese increased (implying decreased meltability) as pH increased [64, 65].

Calcium has a large effect on Mozzarella cheese structure and functionality. Increased amounts of soluble calcium will enhance protein–protein inter- actions, and thus decrease the association of protein with the water phase, to the detriment of meltability [66]. Lower levels of calcium result in decreased numbers of serum pockets and less expressible serum, but increased meltability and decreased firmness. Cheese meltability and the proportion of soluble calcium are reversible over the relatively wide pH range of 4.8 to 6.5 [67, 68]. Good curd flow requires sufficient casein hydration to promote interaction with the water phase. Higher levels of soluble calcium improve protein–protein interactions, reduce protein hydration, promote curd syneresis and, therefore, reduce meltability. The enhanced protein interaction is evident in low-moisture ‘part-skim’ Mozzarella where calcium induces the area occupied by the protein matrix to shrink. This enhanced protein–protein interaction, with concomitant compacting of the protein network, may be due to increased hydrophobic association of proteins through calcium shielding of the casein charges. By this same shielding mechanism, soluble calcium reduces the extent of protein–water interactions (i.e. solvation) [68].

The proportion of soluble calcium increases as pH decreases, suggesting that at low pH the extent of protein solvation and swelling is depressed. Conversely, at high pH the protein matrix will swell and absorb more water, and serum channels will decrease in size. Depletion of calcium causes the protein matrix to become more swollen one day after manufacture [57], indicating enhanced casein solvation. This may also facilitate increased levels of proteolysis. Therefore, the level of calcium is a compromise between the desired functional properties [55]. Unsalted directly acidified Mozzarella has poorer melt properties and a more open protein microstructure compared to salted cheese [69]. Directly acidified Mozzarella curd at pH 5.6 has good stretching properties, becoming excessively soft and fluid–like [43], as more calcium has been lost into the whey, despite the increased proportion of calcium bound to the protein matrix at the higher pH. A lesser amount of total calcium appears to be necessary to promote meltability and flow behaviour [56]. In general, reducing the pH of cheese from 5.8 to 5.4 increases the ratio of soluble–to–colloidal calcium [69, 70].

### 1.2.3 Effect of salt on formation of cheese structure

Salt has three major functions in cheese: it acts as a preservative, contributes directly to flavour, and is a source of dietary sodium. Together with the desired pH, water activity and redox potential, salt assists in cheese preservation by minimizing spoilage and preventing the growth of pathogens. Consequently, the salt level markedly influences cheese flavour and aroma, rheology and texture properties, cooking performance and, hence, overall quality. Many factors affect salt uptake and distribution in cheese and precise control of these factors is a vital part of the cheese making process to ensure consistent, optimum quality [71].

Salt can be incorporated into cheese by direct addition of dry salt to the milled curd pieces, immersing curd blocks in cold brine (usually 8–23 g NaCl 100 $g^{-1}$ water), or a combination of these two processes. When a moulded cheese is placed in brine there is a net movement of $Na^+$ and $Cl^-$ ions from the brine into the cheese as a consequence of the osmotic pressure difference between the cheese moisture and the brine. Consequently, moisture diffuses out through the cheese matrix so as to restore osmotic pressure equilibrium. The

quantity of water lost is about twice the quantity of salt gained, as the size of the $Na^+$ $Cl^-$ ion pair is about twice that of $H^+$ $OH^-$ [71, 72]. As Mozzarella cheese ages, the amount of expressible serum reduces to zero after about 10–20 days. This can be shown by the closing up of voids within the protein matrix in Mozzarella cheese over time. The rate of reduction in expressible serum over time is slower for directly acidified cheese compared to cultured cheese. Increasing salt levels reduces the amount of expressible serum, and this is understood to be caused by increased protein swelling by absorption of cheese moisture. Unsalted Mozzarella cheese has a higher level of expressible serum than salted cheese. Mozzarella cheese with no salt has a more open protein matrix with larger serum pockets compared to a salted cheese. Unsalted cheese with higher amounts of expressible serum will swell over time, but much more slowly than for salted cheese [56, 71].

Cooling the cheese in a brine bath at a lower temperature results in less free oil and more expressible serum, presumably as the hydrophobic interaction responsible for protein interactions is reduced at the lower temperature [71]. Higher sodium chloride concentration and longer brining time also reduce protein porosity at the surface layer. Salt reduces the amount of free oil in aged Mozzarella, possibly by increasing the emulsifying ability of caseins, thereby impairing meltability as there is less free oil to lubricate the protein matrix. With increasing salt, the serum pockets are reduced in size, apparent viscosity increases, but there is no effect on fat globule size or shape in Mozzarella cheese at a point one day after manufacture [56]. For one–day–old Mozzarella cheese, the increased extent of protein swelling induced by a higher salt content does not appear to impact upon free oil formation [71, 72]. The fat globules may be squeezed by the swelling protein matrix immediately after manufacture, but the rate of fat globule coalescence and rupture must be a much slower process, therefore having no impact on free oil formation [56]. Mozzarella cheese with a high salt content of 1.78% is less meltable and less stringy than cheese of equal age with a lower salt content of 1.06%. Insufficient proteolysis due to high salt content can cause a “curdy” texture. The effect of salt on the functionality of cheese is also related to the changes in water-binding capacity [63]. A low salt level and high moisture content can make cheese pasty and off–flavored. A related defect in Mozzarella, described as soft surface defect, occurs when hot plasticized mozzarella curd is placed in cold brine (e.g. <5°C), especially if the brine concentration is low [73].

### 1.2.4 Microbiology of pasta filata cheeses

Microorganisms gain entry into the cheese either by deliberate addition as part of the starter culture or are naturally associated with the ingredients used in cheese production. Thus, the manufacturing technology is central to defining the biodiversity of the cheese flora. The most prevalent microorganisms in cheese, particularly early in ripening, are the starter bacteria [43].

Thermophilic lactic acid bacteria such as *Streptococcus thermophilus*, alone or mainly in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus* or *Lb. helveticus*, are used as starters for most pasta filata cheeses. However, low–moisture Mozzarella for pizza may also be manufactured using mesophilic starters (e.g., *Lactococcus lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*) or some varieties of
Kashkaval cheese also include in the starter formulation *Leuconostoc* sp. and *Lb. casei*; this is because the high temperature used in cheesemaking is more tolerated by thermophilic starters. *Streptococcus thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, and *Lb. helveticus* survive and remain metabolically active when the curd temperature at stretching is ~55 °C. However, the activity of thermophilic starters is substantially decreased at the higher stretching temperature of the curd (e.g., 62–66 °C). Besides, thermophilic starters more easily allow to attain the range of moisture desired for pizza cheese (~48–52%). Nevertheless, in several cases, natural starter cultures have a very heterogeneous composition [43, 45].

In addition to thermophilic lactic acid bacteria, natural whey starter cultures used for the manufacture of high-moisture Mozzarella cheese contain large numbers of mesophilic lactic acid bacteria such as *Lb. plantarum*, *Lb. casei* subsp. *casei*, *Lc. lactis* subsp. *lactis*, and enterococci (mainly *Enterococcus faecium* and *Ec. durans*). A study on a large number of natural whey cultures for Caciocavallo Silano cheese revealed mainly thermophilic lactic acid bacteria, even though the mesophilic *Lc. Lactis* subsp. *lactis* was also present in several preparations. Natural whey cultures for the manufacture of Caciocavallo Pugliese are dominated by strains of *Sc. thermophilus*, *Lb. delbrueckii* ssp., *Lb. helveticus*, *Lb. fermentum*, and *Lb. gasseri* [45].

Modifications in the composition of the microbial population are generally seen during ripening of semihard pasta filata cheeses. Although the thermophilic

lactic acid bacteria from the natural whey cultures dominate during early ripening, Caciocavallo Pugliese harbors a heterogeneous population of non-starter lactic acid bacteria (NSLAB) during late ripening, which is dominated by *Lb. parabuchneri* and *Lb. paracasei* subsp. *paracasei*. *Lactobacillus paracasei* subsp. *paracasei*, *Lb. fermentum*, and*Lb. plantarum* generally dominate in Caciocavallo Silano cheese during late ripening. Ripening of Provolone del Monaco, made without the use of deliberately added starters, is typically characterized by the dominance of thermophilic lactic acid bacteria (*Sc. thermophilus*, *Sc. macedonicus*, *Lb. delbrueckii* spp., and *Lb. fermentum*), together with enterococci and NSLAB of the *Lb. casei* group, especially *Lb. rhamnosus* [45, 46].

The main role of starter cultures during the manufacture of pasta filata cheeses is to synthesize enough lactic acid to demineralize and transform the curd into the state that undergoes stretching in hot water at the target pH (as it was noted above). Furthermore, microbial acidification has to proceed at a rate that allows an adequate syneresis during manufacture to achieve the target moisture content. Rapid acidification allows the manufacturing time to be shortened, which reduces syneresis and enables a high moisture content to be achieved in the final cheese. Starter cultures may be eliminated altogether and replaced by direct chemical acidification of the milk during manufacture of high– or low–moisture Mozzarella, provided that an appropriate level of demineralization in combination with an appropriate pH at stretching is achieved. The secondary role of starters in ripened pasta filata cheeses, including pizza cheese, is concerning secondary proteolysis. Nevertheless, the significance of microbial proteolysis is largely influenced by the temperature of stretching. The synthesis of small peptides and free amino acids (FAAs) by starters is also important in low-moisture Mozzarella because they markedly influence the browning properties of the cheese during melting and baking in pizza making, which is an important functional attribute. Furthermore, Mozzarella cheeses that aremanufactured using thermophilic starters generally have a characteristic yogurt-like note resulting from the synthesis of acetaldehyde by *Sc. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*. Mozzarella that is manufactured without starter cultures through direct acidification will assume the flavor of the chemical compounds used. For example, when vinegar is used as the acidulant, the resulting cheese will possess amild acetic acid flavor note. On the contrary, if citric acid is used, the cheese

will be insipid, due to the lack of flavor other than that arising from milk constituents [45, 46].

### 1.2.5 Changes of protein during cheese ripening

Cheese ripening involves a complex series of biochemical, and probably some chemical events, that leads to the characteristic taste, aroma and texture of each cheese variety [74]. Biochemical changes in cheese during ripening may be grouped into *primary* (lipolysis, proteolysis and metabolism of residual lactose and of lactate and citrate) or *secondary* (metabolism of fatty acids and of amino acids) events. Residual lactose is metabolized rapidly to lactate during the early stages of ripening. Lactate is an important precursor for a series of reactions including racemization, oxidation or microbial metabolism. Citrate metabolism is of great importance in certain varieties. Lipolysis in cheese is catalysed by lipases from various source, particularly the milk and cheese microflora, and, in varieties where this coagulant is used, by enzymes from rennet paste. Proteolysis is the most complex biochemical event that occurs during ripening and is catalysed by enzymes from residual coagulant the milk (particularly plasmin, chymosin) indigenous milk enzymes, starter, and proteinases and peptidases from lactic acid bacteria and, adventitious non-starter microflora and, in certain varieties, other microorganisms that are encouraged to grow in or on the cheese. Secondary reactions lead to the production of volatile flavour compounds and pathways for the production of flavour compounds from fatty acids and amino acids [75, 76, 77].

The chemical composition and biochemical events that occur during ripening of Mozzarella cheese determine its final quality and acceptance, because they have an effect on the functional properties of this cheese variety, which is consumed worldwide. Dry matter, fat content, Ca content, pH evolution during cheese making, and residual levels of lactose and galactose, among others, have been identified as factors affecting Mozzarella cheese texture and functional properties. In addition, pasta filata cheeses represent a special case, because casein molecules are arranged distinctly after the stretching step that takes place during cheese making [66, 78, 79, 80, 81].

Some pasta filata cheeses such as high–moisture Mozzarella and Mozzarella di Bufala Campana are eaten immediately after manufacture without

ripening. On the contrary, low–moisture Mozzarella (pizza cheese) undergoes a brief but essential ripening period (less than 1 month at 4 °C) to develop the desirable functional characteristics. However, significant and characteristic changes in functional properties of Mozzarella cheese take place during the first few weeks after manufacture [82]. Protein, fat, and lactose are hydrolyzed (i.e., proteolysis, lypolysis, and glycolysis, respectively) to varying extents during cheese ripening. Among these, the primary proteolysis in ripening has been defined as the changes in caseins ($\alpha_{s1}$–, $\alpha_{s2}$–, β– and para – κ– casein), is the most important. Proteolysis of α- and β-casein occurs due to any residual rennet from what was added for coagulation, natural proteases, and proteases and polypeptidases from starter or adventitious bacteria. This is essential for cheese flavor development.

The breakdown of proteins first involves the conversion of casein fractions into large peptides. These peptides are later broken down to lower molecular weight products. Breakdown of caseins during proteolysis enables the fat globules enmeshed within the matrix to be released such that they coalesce when cheese is heated, thus increasing meltability [78] and free–oil formation, and it is generally accepted that proteolysis produces the softening of cheese body [83, 84, 85]. The products of secondary proteolysis include the peptides and amino acids that are soluble in the aqueous phase of the cheese.

Proteinases and peptidases from different origin catalyse this process: residual coagulant, milk, starter and non–starter lactic acid bacteria, and adjunct cultures. Lactic acid bacteria (LAB) possess a very comprehensive proteolytic enzymatic system, because of their complex amino acids requirements. LAB requires many amino acids and thus has complex proteolytic systems to liberate the amino acids necessary for growth from the proteins in their environment. A major source of proteolytic enzymes in many cheese varieties is the residual coagulant, often chymosin, that remains trapped in the curd on whey drainage [79, 86].

A short ripening period (~3 weeks) and extensive denaturation of chymosin during the high temperature (~70 °C) stretching step during the manufacture of Mozzarella cheese explain the low level of soluble N. In addition, differences in the action of these proteolytic agents causes in differences in peptide profiles. During the ripening of Mozzarella cheese, $\alpha_{s1}$-CN is produced slowly and γ–

caseins more rapidly, indicating weak chymosin activity and fairly high plasmin activity [75].

The specificity of chymosin on all of the caseins: chymosin cleaves α-casein at seven sites, many of which are located near the hydrophobic C-terminal β-casein, and cleavage of these sites can result in the production of short hydrophobic peptides, which are bitter. The primary site of chymosin action on $\alpha_{s1}$–casein ($\alpha_{s1}$–CN), which results in the production of a small peptide, that is hydrolysed rapidly by starter proteinases. $\alpha_{s2}$-casein is more resistant to hydrolysis by chymosin than is $\alpha_{s1}$–casein; cleavage sites of chymosin on $\alpha_{s2}$–casein are restricted to the hydrophobic regions of the molecule. Although *para*–κ–casein has several potential chymosin cleavage sites, it does not appear to be hydrolysed either in solution or in cheese. Presumably, this reflects the relatively high level of secondary structure in κ-casein compared to the other caseins [75, 76].

The action of plasmin is of particular importance in pasta-filata cheeses and high-cook cheeses. The specificity of plasmin is restricted to peptide bonds, and it degrades the caseins in the order β-casein ~ $\alpha_{s2}$– casein > $\alpha_{s1}$–casein; κ–casein seems to be resistant to the action of this proteinase [86, 87]. $\alpha_{s2}$-casein is also very susceptible to plasmin action and it is likely that the disappearance of this protein, which is often observed in cheese during ripening.

The relative contribution of each proteolytic agent involved in Mozzarella cheese ripening is still discussed. In fact, the cheese making process, both directly and indirectly, determines the relative importance of each proteolytic agent during ripening: it can inactivate enzymes or their inhibitors, and provide a more or less favorable environment for their action [74, 75].

### 1.2.6 Overview of biogenic amines

Biogenic amines (BAs) are aliphatic, alicyclic or heterocyclic organic bases of low molecular weight which arise as a consequence of metabolic process in animals, plants and microorganisms. These amines are found in a variety of foods and beverages whose elaboration includes a ripening or fermentation process [88].

The presence and accumulation of biogenic amines depend on many factors such as availability of free amino acids (level of proteolysis), pH, water activity, salt-in-moisture level, temperature, bacterial density and synergistic effect between microorganisms [88, 89] and primarily, the presence of microorganisms that have amino acid decarboxylase activity such as lactobacilli, enterococci, micrococci and many strains of *Enterobacteriaceae* [89].

The chemical structure of BAs can either be: aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine, phenylethylamine), heterocyclic (histamine, tryptamine). Several authors had classified cadaverine, putrescine, spermine, and spermidine among polyamines [90].

These compounds, such as tyramine, histamine, putrescine, cadaverine, tryptamin and 2–phenyl–ethylamine, have been found in several types of cheese. Cheese is an ideal substrate for biogenic amine formation, since its manufacturing process involves proteolysis, free amino acids liberation, but also the possible presence of decarboxylase-positive microorganisms and the environmental conditions that allow the growth of microorganisms, the decarboxylase enzymes activity (optimal pH, temperature, salt, and water availability), and the presence of suitable cofactors (pyridoxal phosphate) [91, 92].

In addition, particular conditions may also contribute, such as the use of raw material of poor hygienic quality and the length of ripening. Therefore, controlling the hygienic conditions of the raw material and manufacturing processes could avoid contamination with wild decarboxylating microorganisms, which are eventual producers of biogenic amines. The use of milk of high bacteriological quality is critical for amine formation. Therefore, pasteurization is also crucial because it reduces the growth of microorganisms that form amines. High pressure could also be an effective alternative to inactivate microorganisms [93]. After fish, cheese is the next most commonly implicated food item associated with histamine intoxication [94].

The presence of low levels of biogenic amines in cheeses and other foods is not considered a serious risk. However, if normal routes of amine catabolism are inhibited or the amount consumed is large, various physiological effects may result [92]. Several outbreaks of histamine poisoning have occurred following the consumption of cheese, which containing high levels of histamine [94].

“Cheese reaction”, a hypertensive crisis usually accompanied by severe headache, has been observed after ingestion of foods rich in tyramine. Migraine headache has been observed after consumption of cheeses with high levels of tryptamine and 2–phenylethylamine. Biogenic amines may provoke hypertensive crises and even death from cerebral hemorrhage in patients treated with monoamine oxidase inhibitor (MAOI) drugs. Polyamines, such as putrescine, cadaverine, spermine and spermidine can potentiate histamine toxicity [95]. Furthermore, in the presence of nitrite, these amines may form N-nitrosamines, some of which are known to be carcinogenic, mutagenic, teratogenic and embriophatic. The exact toxic threshold of biogenic amines is difficult to determine due to its dependence on the efficiency of detoxification mechanisms of different individuals. However, according to Halász *et al.* [95], 10 mg of histamine in 10 g of sample can cause histamine poisoning; 10-80 mg of tyramine can cause “cheese reaction” (6 mg if patient is receiving MAOI); and 3 mg of 2-phenylethylamine can cause migraine headache [92].

## 2. AIM OF THE DOCTORAL STUDY

The aim of this study is to perform investigations of influence of different technological steps on the distribution of casein molar fraction during pasta filata cheese making process. To achieve this scope the objectives of the current research work are:

- To summarize and understand the structure and role of casein complex on a base of thoroughly performed literature review;
- To evaluate the analyses of raw materials before heating, after heating and following analysis of final products during ripening or, at the end of expiration date;
- To estimate the influence of physicochemical parameters on the degradation of casein complex during technological process;
- To study the degradation of casein complex by Gel Permeation Chromatography (GPC) and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS–PAGE);
- To compare obtained data with the standard fractions of α-, β-, κ-caseins;
- To monitor the influence of heating and extrusion processes on the growth dynamics of microorganisms;
- To estimate the final product by sensory analysis, as a part of a comprehensive evaluation of the impact of cheese curd heating on the quality of the cheese.

# 3. MATERIALS AND METHODS

## 3.1 MATERIALS

### 3.1.1 Samples characteristics

Generally, the manufacturing process of pasta filata cheeses from the Czech Republic dairy-factories includes the following steps. The standardized milk is pasteurized (72-74°C for 20-30 seconds) and then inoculated with starter culture as the milk is pumped into vertical enclosed vats. The inoculated milk is coagulated with rennet. The coagulum is then cut. Part of the whey (20%) is drained off, and water (15-35°C) is added to the mass. The mass is pumped to a pressing vat where it is drained. The fresh-made curd is stretched under hot water (between 60-85°C) in the cooker-stretcher step. In this molten state, the cheese is kneaded until the proper texture is achieved. After this, the curd is forced into a mould. The moulded cheeses undergo further cooling (15-21°C) and salting by immersion in brine solution. In a broad sense, this cooker-stretcher operation is an extrusion process.

Cheese samples were collected from four local dairy-factories (specified as A, B, C, D manufacturers) in southeastern part of the Czech Republic specialized in cow milk pasta filata cheese production. One type of cheese was chosen from each manufacturer. The composition of the final cheese products were taken from the declared data, which were indicated by the manufacturer on the package. The composition of the final cheese products and measured pH values are given bellow:

**A-manufacturer** – Mozzarella (mechanically produced, weight of the final product-1000 g without brine, PA/LDPE packaging, shelf life 14 days, 34 % w/w min.dry matter, 15 % w/w fat, 1% salt, 231 kcal. energy value, pH 5.30);

**B-manufacturer** – Mozzarella (mechanically produced, weight of the final product-350 g without brine, PA/PE vacuum packaging, shelf life 50 days, 45% w/w dry matter, 18% w/w fat, 40% fat in dry matter and 1% salt, without preservative agents, pH 5.20);

**C-manufacturer** – Salted pasta filata cheese (mechanically produced/hand made plaited, weight of the final product-120 g without brine, PA/PE vacuum

packaging, shelf life 60 days, 58% w/w dry matter, 22% w/w fat, 3.2% salt, pH 5.20);

**D-manufacturer** – Smoked pasta filata cheese (mechanically produced, weight of the final product-850 g without brine, PA/PE vacuum packaging, shelf life 60 days, dry matter 52% w/w dry matter, 27% w/w fat, 1.7% salt, pH 5.15).

Samples were collected from several steps during cheese production, which were standard for pasta filata cheese as it is described in Gernigon *et al.* [96]: cheese curds after fermentation or before heating, stretched cheese curds or cheese curds after heating, and final extruded products.

## 3.2 METHODS

### 3.2.1 Chemical analysis

Chemical analysis was applied to the samples of cheese curds from A-manufacturer after fermentation or before heating (shown as Cheese curd I, Cheese curd II). Analysis included the determination of actual acidity (pH), titratable acidity, and water heating temperature. Titratable acidity was recorded as Soxhlet–Henkel degree (SH°).

### 3.2.2 Chromatographic method

A total of twelve samples from manufacturers for chromatographic analysis were selected. From each manufacturer three samples were picked, namely, the cheese curds before heating, cheese curds after heating, and the final products.

GPC analysis was performed using the equipment PLGPC-50 (Polymer Laboratories, Church Stretton, Shropshire, UK) equipped with a PL differential refractometer (DRI) and on–line viscometer detectors (VIS). Analysis was performed with a column set consisting of two columns connected in a series, one TSK GMPWXL (Tosoh Bioscience, Stuttgart, Germany) and one Ultrahydrogel 250 column (Waters Milford, Worcester, Massachusetts, USA). Measurements were carried out at 30 °C; with the mobile phase flow rate of 0.8 ml/min. Aqueous solution (0.1 M $NaNO_3$, 2 g $L^{-1}$ $NaN_3$ and 15% [v/v] acetonitrile) was used as the mobile phase. The columns were calibrated using

polysaccharide pullulan standards (Polymer Laboratories, Church Stretton, UK) with molecular weights ranging from 180 to 788 000 g/mol. A 100 μl injection loop was used for all measurements. Universal calibration was applied to determine the molecular weight from the DRI and the VIS signal. Data processing was controlled by Cirrus GPC, Multi Detector Software (Polymer Laboratories, Church Stretton, Shropshire, UK). All characteristics were given in comparison with acid and rennet caseins.

Prior to measurements, the samples of cheese curds and cheeses were prepared by disintegrating of 0.1 g over 5 minutes, dissolution in 5 ml of 1 M $CH_3COONa$, stirring and heating (under 60 °C). A slightly opalescent solution was obtained by the partial dissolution of casein. The samples were filtered after cooling through a 0.45 μm Chromafil PP/PET filter. The filtrate was diluted with distilled water in 50 ml volumetric flask. All measurements were performed twice. Elution conditions were optimized.

Compared to discrete molecules which have well-defined molecular weights, polymers are composed of hundreds to thousands of chains of different molecular weights that result in characteristic molecular weight distribution (MWD). In order to describe MWD, moments or statistical averages of the distribution are calculated. In most cases, number-average $M_n$ and weight-average $M_w$ molecular weights are determined as the characteristics describing MWD. The magnitude of $M_n$ is sensitive to the presence of low molecular weight species and $M_w$, on the other hand, indicates changes in high molecular weight component. The width of MWD can be characterized by the polydispersity index (PDI), simply determined as the ratio of $M_w/M_n$. In addition, $M_w$ and $M_n$ values can be statistically calculated from gel permeation chromatography measurements [97].

Weight average molecular weight is defined as:

$$\overline{M}_w = \frac{\sum(w_i * M_i)}{\sum w_i} = \frac{\sum N_i * M_i^2}{\sum N_i * M_i} \qquad (1)$$

Number average molecular weight is defined as:

$$\overline{M}_n = \frac{\sum w_i}{\sum N_i} = \frac{\sum N_i * M_i}{\sum N_i}, \text{ where} \qquad (2)$$

$w_i$ - is the weight of molecules with molecular weight $M_i$

$N_i$ - is the number of i-th molecules with molecular weight $M_i$

### 3.2.3 Electrophoretic analysis

The electrophoretic analysis was carried out on seventeen individual samples. Samples of pasteurized milk, cheese curd before heating, cheese curd after heating, final product, and final products after one month of ripening were acquired from A-manufacturer. Each samples of cheese curds before heating, cheese curds after heating, final products, and final products after one month of ripening were obtained directly from B, C, and D-manufacturers.

The protein profiles of the samples were studied by the sodium - dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Sambrook *et al.* [98] on the 5% stacking and 15% resolving polyacrylamide gel slabs. The electrophoresis was performed in a vertical dual plate unit (Owl Separation Systems, Portsmouth, New Hampshire, USA) with a power supply of MP-500P (500V). The 0.8 mm thick glass plates were used.

Samples of the cheese curds and cheeses were prepared by homogenizing 5 g samples in 5 mL of deionized water in a homogenizer Stomacher (Seward Ltd., Worthing, West Sussex, UK) for 10 min. The suspensions were centrifuged in a universal centrifuge (Hermle Z 300 K, Wehingen, Germany) at 4°C and 3000 × g for 25 min. Supernatant fluids were diluted as described by Lazárková *et al.* [99]. The mixtures were heated in the dry block heating thermostat (Bio TDB-100, Riga, Latvia) at 100°C for 10 min. The volume of 15 µl samples and standards were applied under the cathodic buffer to the gel.

Electrophoresis was carried out at room temperature using a voltage stepped procedure: electric current was kept constant (40 mA) until the samples completely left the stacking gel. Then the electric current was increased and

maintained constant (60 mA) until the tracking dye reached the bottom of the gel. Electrophoresis was stopped after four hours.

Immediately after electrophoresis, the gel was removed from the plates and placed in a fixative solution (10% trichloroacetic acid) at room temperature. After 20 min, the fixative solution was replaced by a staining solution contaning 0.25% Coomassie Blue R-250, 50% [v/v] methanol and 10% [v/v] acetic acid and the gel was left on the Multi-Shaker PSU 20 (Biosan, Riga, Latvia) for 30 min. Destaining solution included 25% [v/v] methanol and 10% [v/v] acetic acid. The gel was destained for two hours. The reagents for sample preparation and electrophoresis were supplied by Serva (Heidelberg, Germany) and those for staining from Lach-Ner (Neratovice, Czech Republic). The molecular weight Protein Marker (Broad Range (2-212 kDa), Ipswich, Massachusetts, USA) was used for standardization of the relative electrophoretic mobility of the proteins. The mixture of molecular weight Protein Marker consist of: aprotinin from bovine lung - 6.517 kDa, lysozyme from egg white – 14. 313 kDa, triosephosphate isomerase from *E.coli* – 27 kDa, serum albumin from bovine, myosin from rabbit muscle – 212 kDa. The standards of $\alpha$-, $\beta$- and $\kappa$-caseins (Sigma Aldrich, St. Louis, USA) were used as controls.

The documentation system of Gene Snap was used to photograph the polyacrylamide gel slabs and Gene Tools software (both from Syngene, Cambridge, UK) was used to analyze images of the molecular weight of protein-banding patterns.

### 3.2.4 Microbiological analysis

Microbiological analysis was conducted with eighteen samples from each manufacturer throughout the technological process: the cheese curds before heating, after stretching in hot water, and after packaging (final product). Moreover, final products from A and C- manufacturers were stored in 6 ± 2°C according the cheese type: A – 3 weeks, C – 3 months.

Microbiological analysis was prepared according to [100-102]. One millilitre of milk was added into 9 ml of sterile saline solution and in case of solid samples (curd after fermentation, stretched curd, final extruded products) five grams were added into 45 ml of sterile saline solution (1% w/v sodium

chloride) and homogenized with a blender (Stomacher, Seward Ltd., UK). Appropriate ten times dilutions were plated on Plate Count Agar (Himedia Laboratories, India) for determination of total counts of aerobic mesophilic bacteria (30±1°C/48h) and aerobic psychrotrophic bacteria (8±1°C/10 days), on Violet Red Bile Agar (Himedia Laboratories, India) for determination of coliform bacteria (37±1°C/24h), on Chloramphenicol Yeast Glucose Agar (Himedia Laboratories, India) for determination of yeasts (20±1°C/5 days), on MRS Agar (Himedia Laboratories, India) for determination of lactobacilli (37±1°C/48h/5%$CO_2$), on M17 Agar (Oxoid, England) supplemented with 1% glucose and 1% lactose for determination of lactic streptococci (37±1°C/48h). Microbial counts were then expressed as log $CFU.g^{-1}$.

### 3.2.5 Biogenic amines analysis

Biogenic amines (BAs) were observed in final cheese products. The samples characterization is given in Table 4. Extraction of BAs from cheese was performed according to Buňková *et al*. [103]. Analysis of BAs was carried out by using ion-exchange chromatography (AAA400 Amino Acid Analyzer; Ingos, Prague, Czech Republic). The samples were separated and determined using the conditions described by Buňková *et al*. [104]. Each sample was analyzed twice. The reagents for sample preparation, separation and detection were obtained from Ingos (Prague, Czech Republic). Standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Table 4.** Characterization of the selected samples.

| Samples | Designation | Storage time (days) |
|---|---|---|
| Final product from A-manufacturer | $A_f$ | 17 |
| Final product from B-manufacturer | $B_f$ | 13 |
| Final product from C-manufacturer | $C_f$ | 14 |
| Final product from D-manufacturer | $D_f$ | 15 |

### 3.2.6 Sensory analysis

Six samples of final product of pasta filata cheeses were chosen for the sensory analysis. Samples were stored under refrigeration (4°C) and were held at ambient temperature for about 1 h before sensory evaluation. Specifications of the samples are given below:

A1 – Mozzarella I, one day after manufacturing (A-manufacturer)
B2 – Mozzarella II, one day after manufacturing (B-manufacturer)
C3 – Mozzarella I, seven days after manufacturing (A-manufacturer)
D4 – Mozzarella I, 30 days after manufacturing (B-manufacturer)
E5 – Salted pasta filata cheese, one day after manufacturing (C-manufacturer)
F6 – Smoked pasta filata cheese, 22 days after manufacturing (D-manufacturer)

Sensory analysis was specifically focused on the evaluation of the dominant sensory profile, which is, evidently, the flavor of cheese and as an additional profile the rheological properties (consistency) and the color of cheese were evaluated.

Sensory evaluation was performed in order to:

- Choose from a range of sensory evaluated samples the most suitable ones;
- Verify the impact of used technology, the type of cheese ripening and to define sensory characteristics (profiles).

For sensory evaluation ordinal preference test, paired preference test and evaluation of sensory characteristics with verbal expression were used [105, 106].

Ordinal preference test was chosen in order to select the best sample based on preferences of trained sensory panel of assessors. The results can be useful for the manufacturer and serve as a criterion in estimation of product popularity [107].

Evaluation of sensory characteristics was done by descriptive method (verbal). Due to discrepancies of analysed samples (different types of cheeses, different ripeness) a complementary method of descriptive sensory analysis was

used. Revealing ability of this method is relatively high when the panel of evaluators is composed of trained assessors [108].

### 3.2.7 Statistical analysis

The electrophoretic data were exposed to a hierarchical cluster analysis (Euclidean distance measure; linking method–average between groups). The statistical evaluation was done using the STATISTICA Cz StatSoft Version 6 software (StatSoft Ltd, Prague, Czech Republic).

The data obtained by microbiological analysis (averages of microbial counts in log CFU.$g^{-1}$) were statistically analyzed. The T-test using software Statistica for Windows (STATISTICA Cz, StatSoft Version 6, StatSoft Ltd, Czech Republic) was used to evaluate the statistical differences ($P^3 0.05$) between cheese curd and final cheese samples during technological steps, eventually during storage.

The Student's t-test was used for comparisons of biogenic amines data. The standard deviations (SD) were calculated.

The Friedman's test was used for sensory evaluated samples to determine significant differences ($P < 0.05$).

# 4. RESULTS AND DISCUSSION

## 4.1 Chemical analysis

Chemical analysis including the determination of actual acidity (pH) and titratable acidity (°SH) were performed on the samples from A-manufacturer. The results of chemical analysis are listed in Table 5. pH is one of the major manufacturing parameters that plays an important role in the stretching and kneading ability of curd in hot water during cheese making, affecting the functional properties of cheese [70].

**Table 5.** Dependence of water temperature on actual acidity and titratable acidity of cheese curds (mean values)

| Cheese curd I [°SH] | Cheese curd II [°SH] | Cheese curd I [pH] | Cheese curd II [pH] | Water temperature [°C] |
|---|---|---|---|---|
| 105 | 106 | 4, 94 | 4, 98 | 75 |
| 85 | 78 | 5, 09 | 5, 15 | 85 |
| 108 | 90 | 4, 97 | 5, 03 | 73 |
| 106 | 103 | 5, 02 | 5, 06 | 71 |
| 95 | 77 | 5, 02 | 5, 15 | 73 |
| 98 | 89 | 5, 02 | 5, 08 | 71 |
| 104 | 102 | 5, 05 | 5, 09 | 78 |
| 95 | 90 | 5, 07 | 5, 10 | 77 |
| 102 | 97 | 4, 98 | 5, 19 | 72 |
| 82 | 85 | 5, 16 | 5, 17 | 86 |
| 101 | 97 | 5, 09 | 5, 15 | 84 |
| 60 | 68 | 5, 25 | 5, 20 | 85 |
| 88 | 92 | 5, 06 | 5, 25 | 75 |
| 94 | 85 | 5, 11 | 5, 18 | 78 |
| 73 | 82 | 5, 20 | 5, 10 | 70 |

Reducing the pH during cheese making is reported to increase the level of loss of calcium from the curd and increases the extent of fusion of para-casein particles. It is generally accepted that pH, titratable acidity values influence the ability of curd to plasticize in hot water or hot dilute brine [57, 66]. In addition, the higher actual acidity the lower temperature of heating. Dependence of water temperature on titratable acidity is presented in Figure 4.

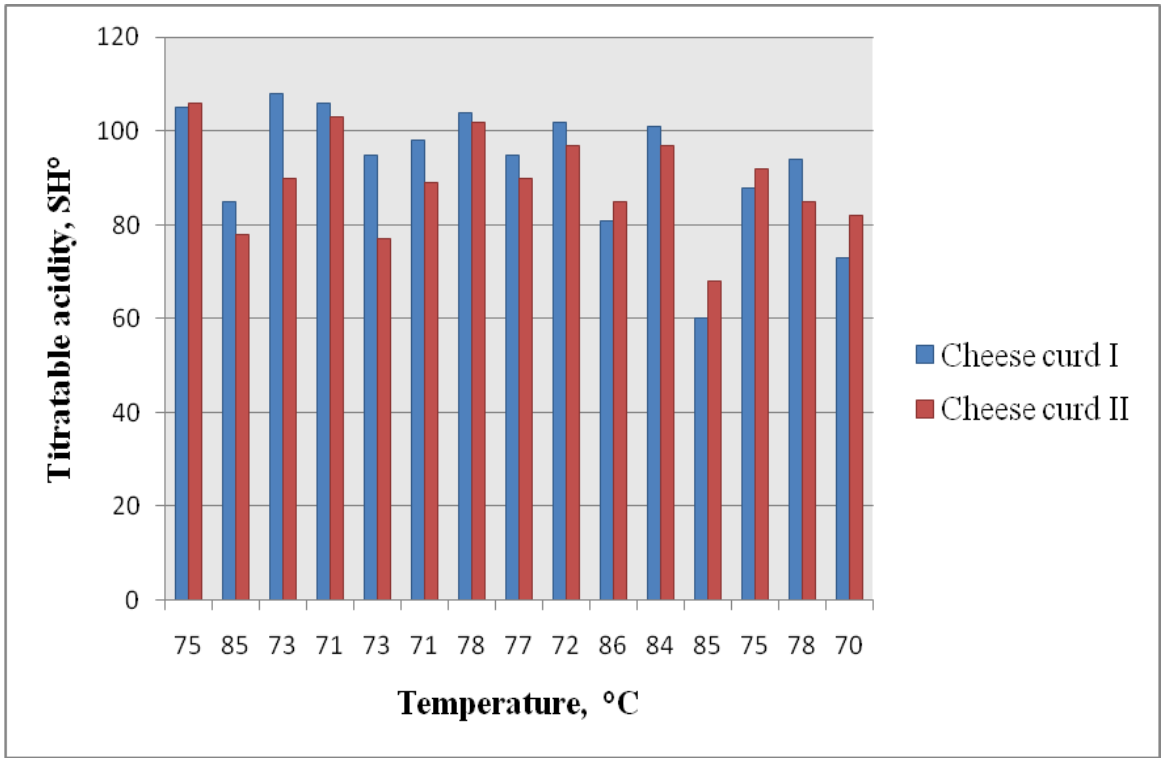


**Figure 4.** Dependence of water temperature on titratable acidity for cheese curds I, II.

The curd becomes progressively less smooth and more lumpy with increasing pH. However, curd may be plasticized successfully at a higher pH (e.g., 5.6) [64, 66].

Dependence of water temperature on actual acidity is shown in Figure 5.

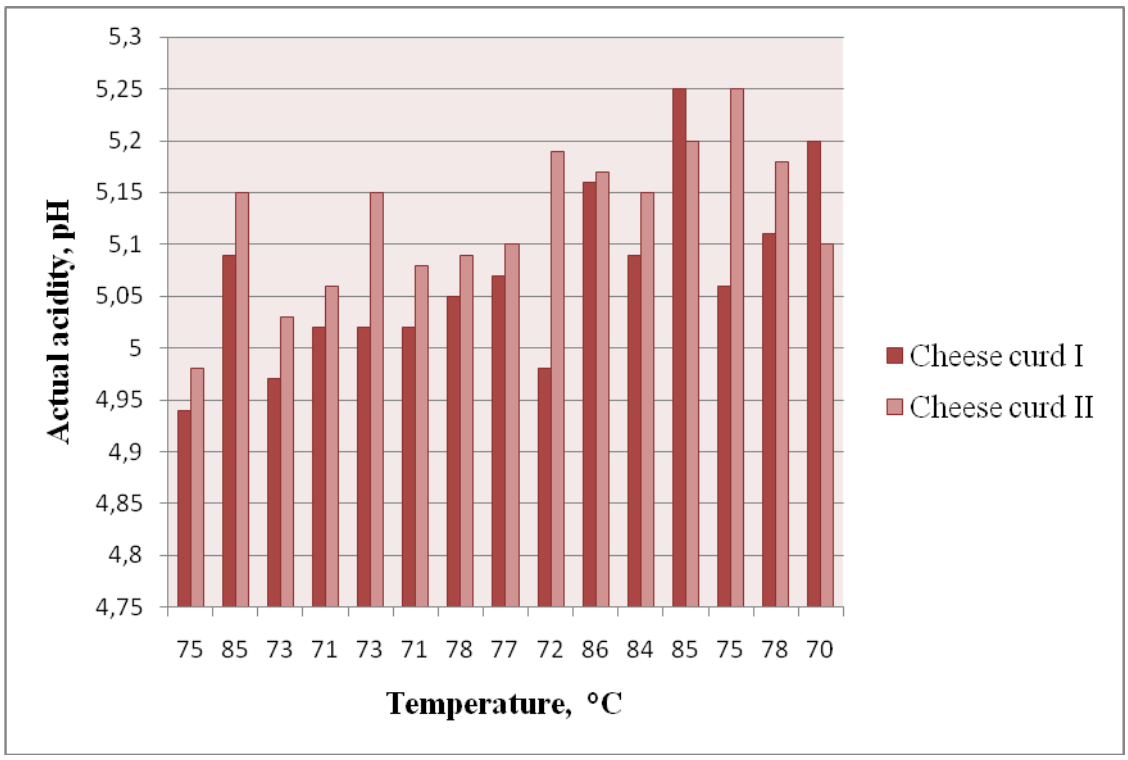


**Figure 5.** Dependence of temperature on actual acidity for cheese curds I, II.

### 4.2 Chromatographic analysis

GPC is an analytical tool routinely used for characterization of molecular weights distribution of polymers, complex foods such as milk and dairy products. If equipped with a viscosity detector, GPC can be with advantage used for absolute molecular weight determination.

Using GPC, both weight average molecular weight $M_w$ and number average molecular weight $M_n$ were obtained. Results from chromatographic analysis are given in Table 6.

It should be noted that the values of $M_w$ and $M_n$ (Table 6) vary significantly from the data reported in the literature [5, 109]. The authors note, that the milk proteins are quite small molecules, with the molecular mass of 19000-25000 g/mol, consequently, these values should be taken as relative. The $M_w$, as well as $M_n$ values increase in the sample of cheese curd after heating compared to sample of cheese curd before heating (A-manufacturer). The increase of those values of the sample cheese curd after heating were occurred, most probably, by

influence of temperature action, which takes place during technological process. Subsequently, the values of the final product cheese from A-manufacturer are decreased. Similar changes were observed in samples from B- and D-manufacturers. Meanwhile, the activation and additional aggregation of casein complex can occur, which may result in lower solubility, lower isolation, and higher detection of molecular weights.

**Table 6**. Values of weight average molecular weight $M_w$ and number average molecular weight $M_n$ measured for selected samples.

| | Samples | $M_n$ [g*mol$^{-1}$] | $M_w$ [g*mol$^{-1}$] |
|---|---|---|---|
| **A** | Cheese curd before heating | 42000 | 82000 |
| | Cheese curd after heating | 97000 | 245000 |
| | Final product | 25000 | 46000 |
| **B** | Cheese curd before heating | 27000 | 42000 |
| | Cheese curd after heating | 138000 | 250000 |
| | Final product | 19000 | 34000 |
| **C** | Cheese curd before heating | 43000 | 78000 |
| | Cheese curd after heating | 69000 | 108000 |
| | Final product (salted cheese) | 161000 | 211000 |
| **D** | Cheese curd before heating | 47000 | 85000 |
| | Cheese curd after heating | 105000 | 168000 |
| | Final product (smoked cheese) | 50000 | 70000 |

On the other hand, the data obtained for $M_w$ and $M_n$ of the final product cheese from C-manufacturer did not decrease, as occurred in previous samples, but rather increased. Those results of samples molecular weight could have been influenced by insufficient heating or dissolving of the samples in the preparation procedure and thus the required release of caseins was not reached.

The elution profiles of the analyzed samples (A-D manufacturers) are shown in Figure 6. All measurements were compared with samples of standard caseins.

The elution profiles were characterized by retention time ranged between 17-19.5 minutes. While the peaks of low molecular weight compounds are situated between 21-28 minutes in all chromatograms.

For consideration the latitude distribution of the molecules of analyzed samples the polydispersity index (PDI) was used. PDI of A was varied from 1.9 (cheese curd before heating) to 2.5 (cheese curd after heating) and dropped to 1.8 for final product in the samples. Together with molecular weight lowering, the PDI decreased from 1.8 to 1.5 and from 1.8 to 1.3 for B and C, respectively. The PDI for D decreased from 1.8 to 1.4. PDI indicates thus narrowing of molecular weight distribution during analysis.

In addition, the shape of differential distribution curves provides a quantitative characterization of the molecular weight of macromolecules that are present in the analyzed samples. It was shown, the sample of final product of cheese from B-manufacturer had a decreased values of $M_w$ and $M_n$ in comparison with a sample of cheese curd before heating, as indicated by a shift of differential distribution curve to the region with low molecular weights (Fig. 7, 1-3). Oppositely, the differential distribution curve has shifted to the region with higher molecular weight for sample of cheese curd after heating. As can be seen (Fig. 7, 4-6), the differential distribution curve for a sample of cheese curd before heating has monomodal peak. Whereas, the distribution curve of cheese curd after heating and final product have partially separated peaks of bimodal distribution.

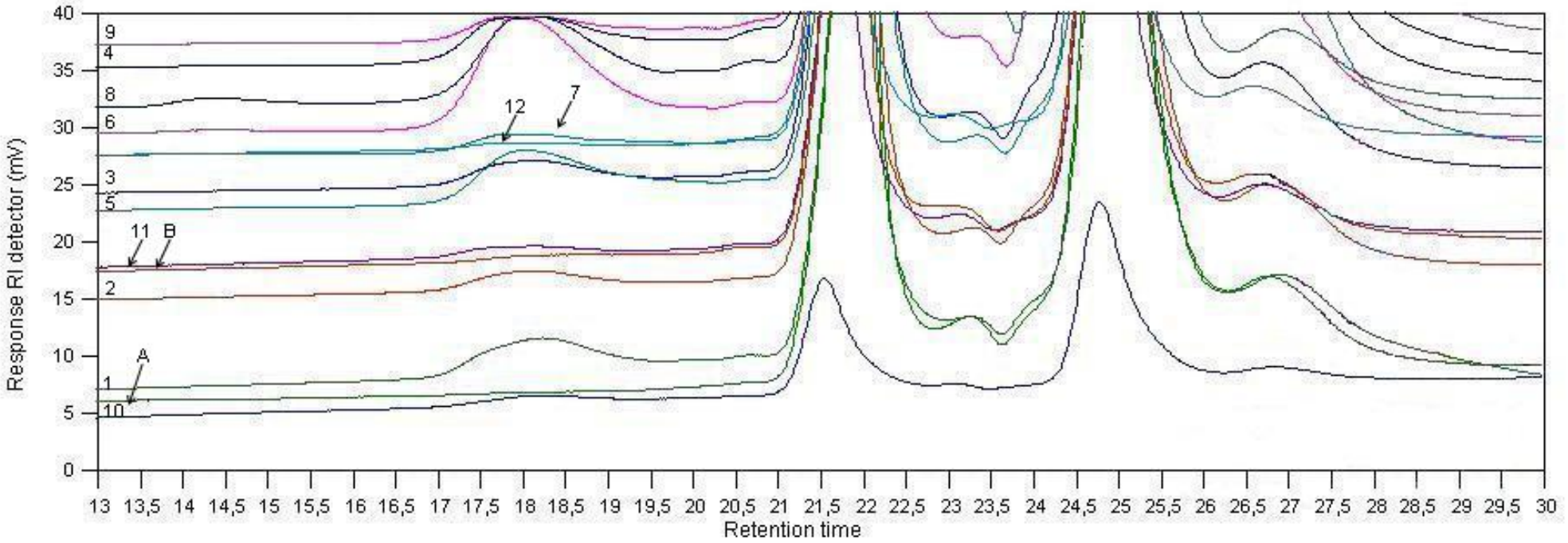


**Figure 6.** GPC profiles: 1–cheese curd before heating **(A)**; 2–cheese curd after heating **(A)**; 3–final product **(A)**; 4–cheese curd before heating **(B)**; 5–cheese curd after heating **(B)**; 6–final product **(B)**; 7–cheese curd before heating **(C)**; 8–cheese curd after heating **(C)**; 9–final product (salted cheese) **(C)**; 10–cheese curd before heating **(D)**; 11–cheese curd after heating **(D)**; 12–final product (smoked cheese) **(D)**; A–rennet casein; B–acid casein.

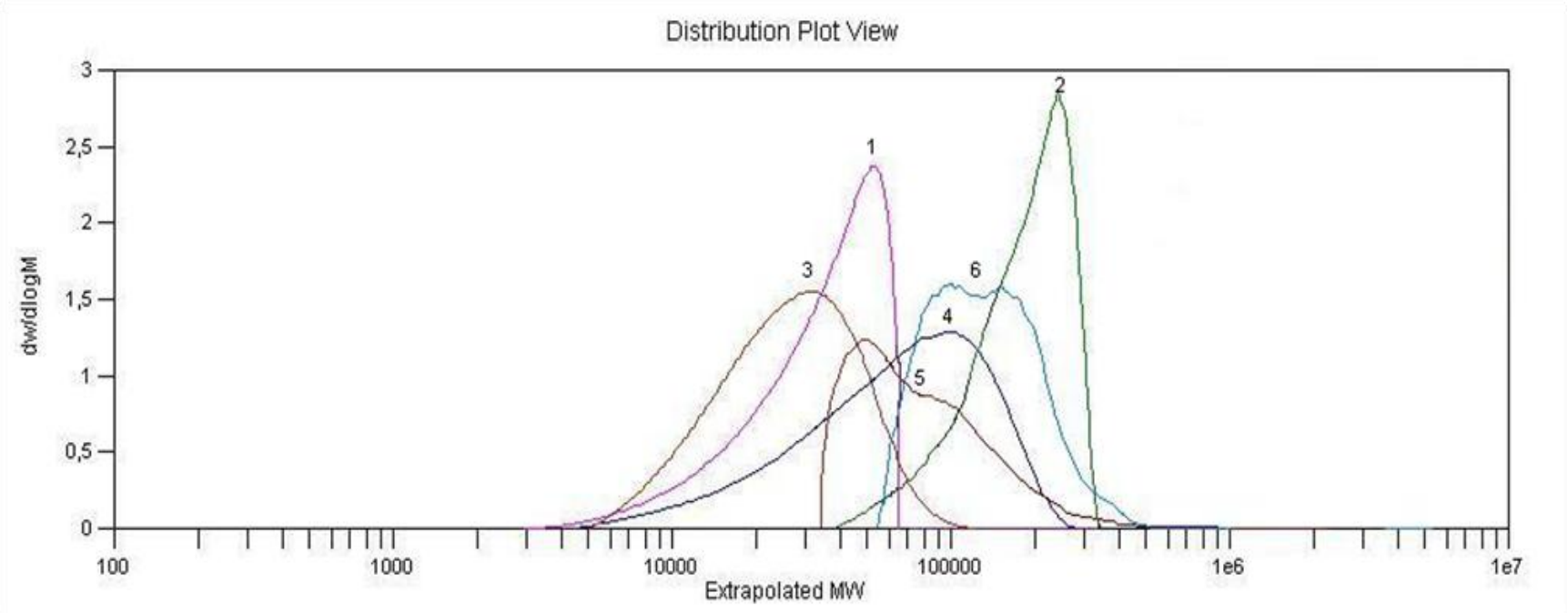


**Figure 7.** Differential distribution curves recorded for samples B, C-manufacturers. 1–cheese curd before heating **(B)**; 2–cheese curd after heating **(B)**; 3–final product **(B)**; 4–cheese curd before heating **(C)**; 5–cheese curd after heating **(C)**; 6–final product (salted cheese) **(C)**;

### 4.3 Electrophoretic analysis

Protein separation by SDS-PAGE is a useful method to estimate relative molecular mass, to determine the relative abundance of major proteins in a sample.

SDS-PAGE analysis showed the electrophoretic patterns of the samples, as well as their extent and differences. The banding protein patterns obtained by SDS-PAGE for samples from A-manufacturer are shown in Figure 7. The protein profiles of pasteurized milk (Fig.8, lane 1) are characterized by the presence of high molecular weight (m.w.) fractions and contains six major bands (m.w. range from 14.3 to 66.4 kDa). The sample of cheese curd before heating (Fig.8, lane 2) showed the two more bands: 80 kDa-bands, and the band with low m.w. 15 kDa. On the other hand the intense 27.0 kDa-band completely disappeared. From sample of cheese curd after heating (Fig.8, lane 3) it is obvious, that some part of proteins is denatured during stretching-extruder process. The final product from A-manufacturer (Fig.8, lane 4) is similar to cheese curd after heating (Fig.8, lane 3) with the exception of fraction 15 kDa.

Sample of final product after one month of ripening (Fig.8, lane 5) in strict contrast to previous samples showed electrophoretic pattern with intense bands in the region of the casein degradation during the storage period and arising of more fractions with low m.w. range from 12 to 25 kDa.

The calculated molecular masses of $\alpha$-, $\beta$- and $\kappa$- caseins are 34 kDa, 30 kDa, 27 kDa, respectively (Fig. 8, lane 6-8). These values represent a discrepancy with casein weights published in literature [5, 16, 109], where described molecular masses range from 19 to 25 kDa. Generally, the apparent molecular masses of caseins are overestimated by SDS-PAGE, since the individual caseins bind larger amount of SDS than other proteins [110]. The fractions of m.w. 27.0-34.6 kDa are present in all samples (Fig.7). Lanes 1-5 show intense bands in the region of $\alpha$- and $\beta$- caseins.

The protein profiles of the samples from B- and C-manufacturers are shown in Figure 9. These electrophoretic patterns of cheese samples from B-manufacturer showed that the protein profile surprisingly did not vary during the stretch-extruder process. Nevertheless, protein profile undergoes notable changes during ripening of the cheese. Protein profile of pasta filata cheeses during technological process is characterized by the presence of a high m.w. fraction and according to the m.w.

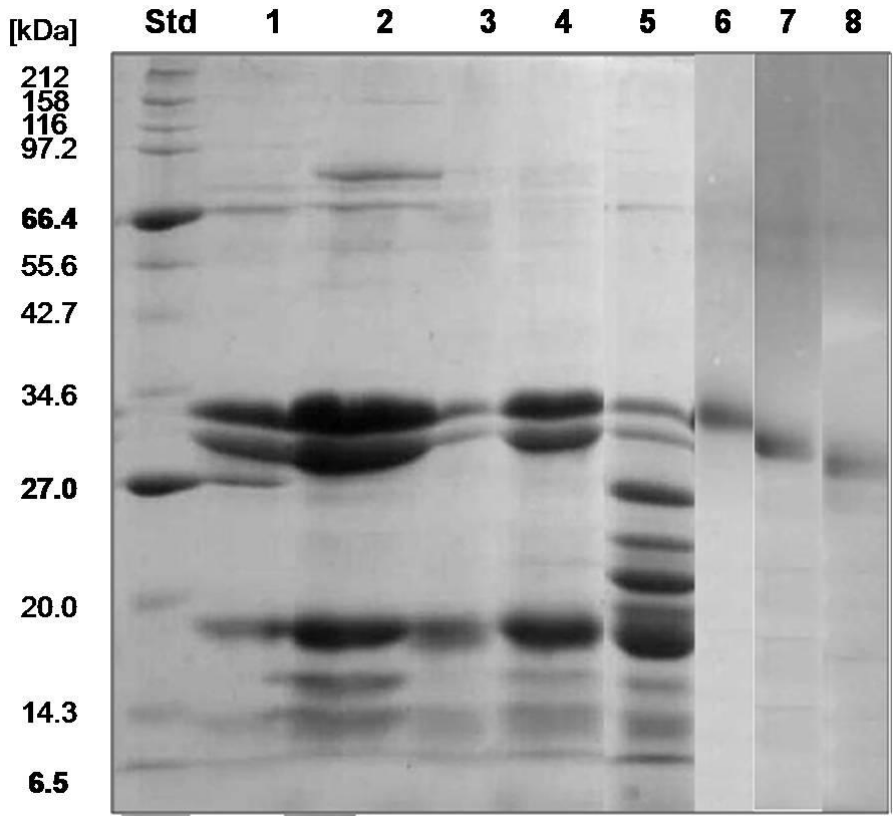


**Figure 8.** SDS-PAGE protein profiles of samples from A-manufacturer. Std-Molecular weight standards (kDa). 1-pasteurized milk; 2-cheese curd before heating; 3-cheese curd after heating; 4-final product; 5-final product after one month ripening; 6-$\alpha$-casein, 7-$\beta$- casein, and 8-$\kappa$-casein.

standard in the range of 66.4-97.2 kDa and two more fractions with low m.w. 14.3-20 kDa. It was determined that final product from B-manufacturer after one month of ripening revealed two new bands with m.w. 15 and 25 kDa. These low weight fractions could refer to protein degradation.

The electrophoregram of samples from C-manufacturer (Fig. 9, lane 5) showed weak 80 kDa-band, which remained present during production process and ripening, although, protein profiles undergo change and all bands bigger than 20 kDa are weaker after heat treatment (lane 6). As well as it was observed in ripened cheese from B-manufacturer, the 15 and 25 kDa bands were formed in C type final product after one month of ripening (lane 8).

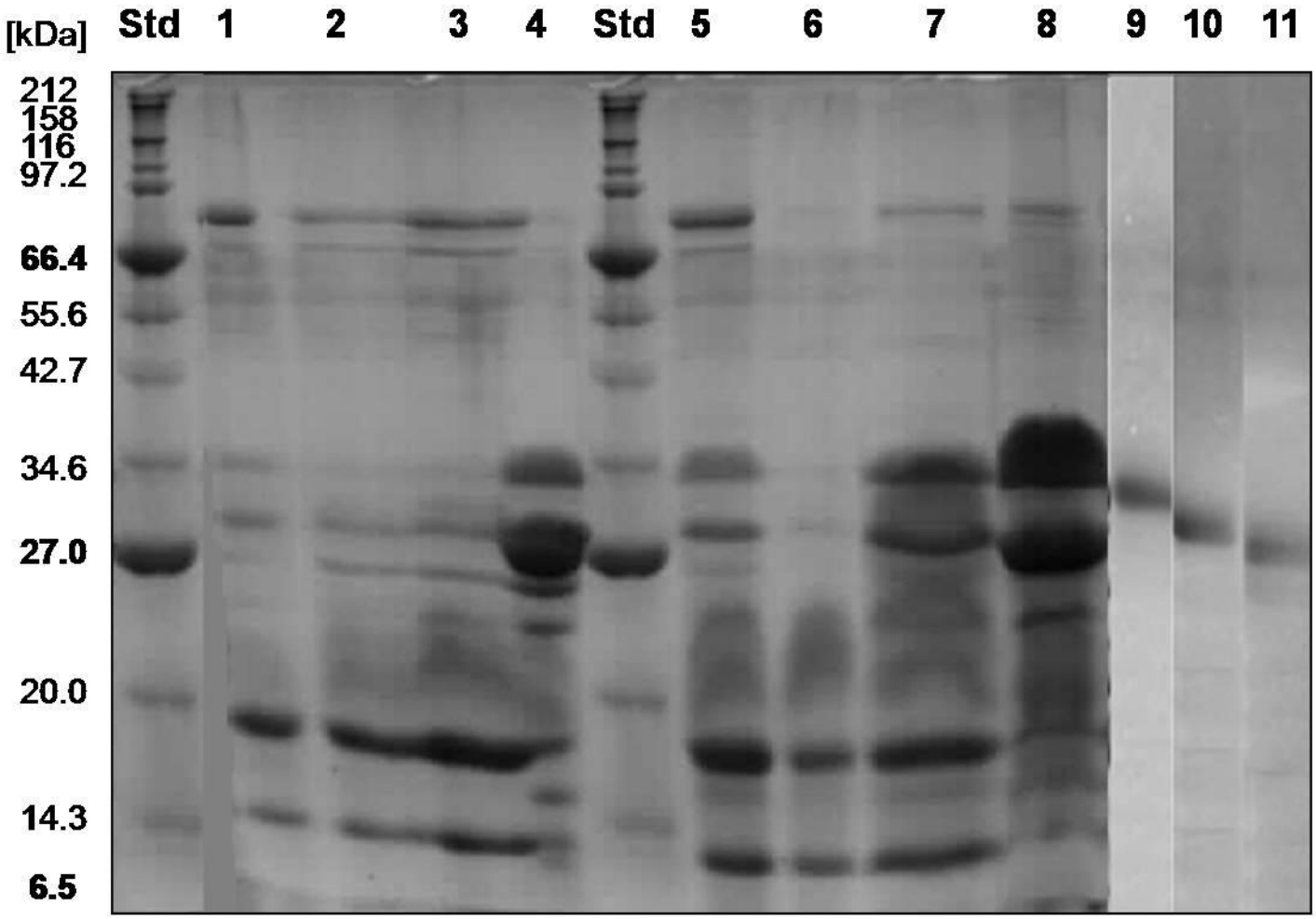


**Figure 9.** SDS-PAGE protein profiles of samples from B and C manufacturers. Std-Molecular weight standards (kDa). M2: 1-cheese curd before heating 2-cheese curd after before heating; 3-final product; 4-final product after one month ripening; M3: 5-cheese curd before heating; 6-cheese curd after before heating; 7-final product; 8-final product after one month ripening; 9-$\alpha$-casein, 10-$\beta$- casein, and 11-$\kappa$-casein.

Figure 10 demonstrated the electrophoretic patterns of the smoked cheese samples from D-manufacturer. The samples of raw material before heating the curd include almost the same casein fractions likewise in samples from A-, B-, and C-manufacturers. The final product (smoked cheese) from D-manufacturer contains similar protein fraction 25 kDa as compared to the sample cheese after one month of ripening from C-manufacturer. It can be concluded that technological process of D cheeses production and following ripening did not lead into any basic differences in protein profiles.

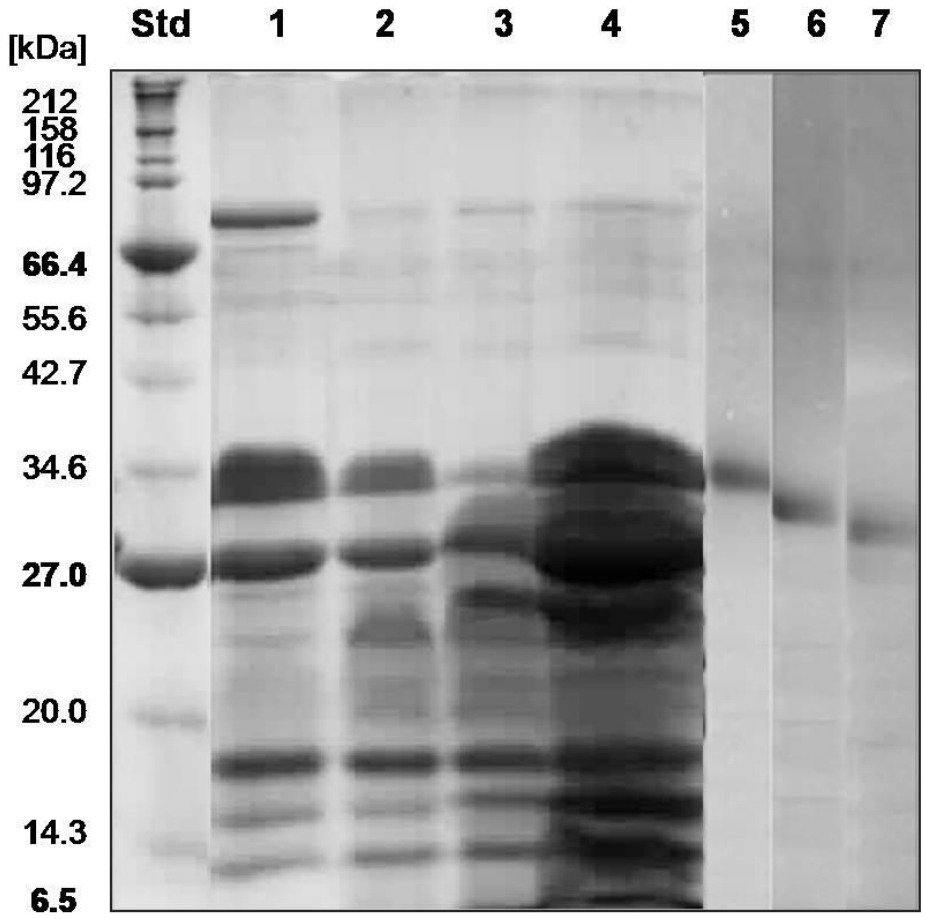


**Figure 10.** SDS-PAGE protein profiles of samples from D-manufacturer. Std-Molecular weight standards (kDa). 1-cheese curd before heating; 2-cheese curd after before heating; 3-final product (smoked cheese); 4-final product after one month ripening; 5-$\alpha$-casein, 6-$\beta$- casein, and 7- $\kappa$-casein.

The definite degradation of casein complex, hydrolysis of casein and major peptides were not considered in this study. However, it was demonstrated that the

molecular mass changes in protein profiles in various stages of the production process compared to casein standards. According to the SDS-PAGE results the diversity in protein profiles of pasta filata cheeses vary depending on the stage of technological process and type of cheese.

It is well known, that the meaningful changes in the protein profiles of samples are usually attributed to the proteolysis. The initial breakdown of caseins to large peptides (i.e. primary proteolysis) in pasta filata cheeses occurs primarily through the action of the coagulant on α- and β-caseins when chymosin or coagulants is used in cheese making [111].

The starter culture may also hydrolyse intact β-casein to a small but significant extent during ageing. However, the principal contribution of the starter culture to casein breakdown occurs in the form of secondary proteolysis (i.e. the subsequent hydrolysis of primary peptides to smaller peptides and free amino acids). Thus, a proteolytic synergy occurs between the coagulant and the starter as it does in most other cheese varieties [45].

The rates of both primary and secondary proteolysis in pasta filata cheeses can vary greatly depending on the proteolytic activity of the coagulant, the extent to which the coagulant and starter culture are heat inactivated during stretching [112], pH, moisture levels of curd, ripening temperature, and humidity [113].

The significant changes in samples of final product from A-manufacturer were observed after one month of ripening with high degree of maturity; whereas the extent of proteolysis was very limited in samples of the final product from B-manufacturer. Presumably, this can be the result of low microflora activity and indigenous enzymes in cheese, as have been pointed out [113].

The final products from C-manufacturer contain a high percentage of salt (brine-salted cheese), in contrast to the other samples. It has been reported that salt does not affect the amount of soluble peptides [77] or the extent of hydrolysis of $\alpha_{s1}$-casein or β-casein [45]; however, in [114] was shown that salt enhances proteolysis. Hydrolysis of $\alpha_{s1}$-casein increases over the first 50 days of ripening; nevertheless, β-casein remains relatively unchanged during this period [115]. Other researchers have found no effect on proteolysis at different salt levels during ripening [45].

It has been investigated, that the very significant changes in protein profile of final product from C-manufacturer have not occurred after one month of ripening. The electrophoretic bands in sample after one month of ripening from D-

manufacturer are characterized by the hydrolysis of proteins and thereby, the bands disappeared in position of high molecular weight.

The protein profiles of the samples obtained by SDS-PAGE method were statistically analyzed by the means of cluster analysis. The results are presented in the form of dendrogram in Figure 11. The cluster analysis revealed that the samples can be classified into three distinct clusters.

The samples of final products at the day of manufacturing predominate in the first cluster, with the exception of cheese curd before heating from D-manufacturer (Fig. 10, 14). The primary function of enzymes in the cheese manufacturing is to coagulate the milk to produce cheese curd. Several authors [5, 116, 117] have pointed out, that some residual rennet may be retained in the curd after draining. This may affect changes of proteins and increase proteolysis during maturation. Therefore, most probably, the protein profile of cheese curd before heating from D-manufacturer is present in this cluster.

The second cluster is formed by the samples of cheese curd before heating and cheese curd after heating for almost all types of analysed pasta filata cheeses. In addition, the sample of the final product from B-manufacturer belongs to this cluster. The primary proteolysis may differ for different pasta-filata varieties depending on the stretching temperature [118]. High stretching temperature used during the manufacturing of cheese from B-manufacturer (70°C) results in limited residual coagulant activity and therefore, proteolysis proceeds slowly and so the current sample is location in this cluster.

Final cheeses after one month of ripening from A, B-manufacturers (Fig.11. 5, 9) and one sample of fresh final product from A-manufacturer form the third cluster. In contrast, two more samples of cheese curd after heating from C- and D-manufacturers belong to this cluster as well. It should be noticed that, the manufacturing process of C- and D-dairy-factories involves immersion of cheese curd in cool water after heating and further holding in brine solution (20 min at D and 18 hours at C) before packaging. De Angelis *et al*. [45] concluded that owing to the persistence of sodium chloride gradient between the center and rind of the curd may influence proteolysis during cheese ripening as well as changes in protein profiles. In this case, it can be assumed that these samples belong to one cluster.

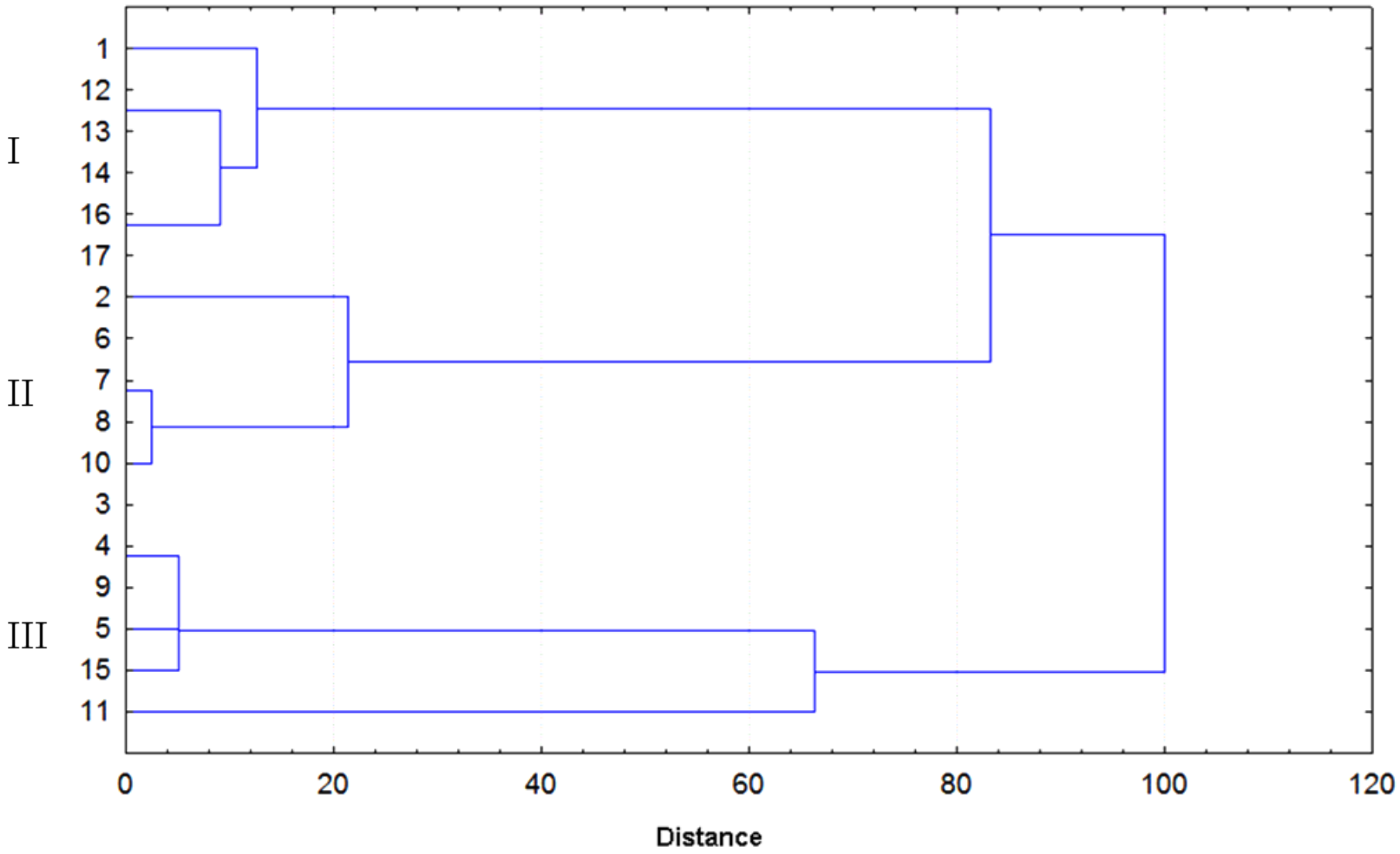


**Figure 11.** The result of cluster analysis. Samples: 1-milk after pasteurization **(A)**; 2-cheese curd before heating **(A)**; 3-cheese curd after heating **(A)**; 4-final product **(A)**; 5-final product after one month ripening **(A)**; 6-cheese curd before heating **(B)**; 7-cheese curd after heating **(B)**; 8-final product **(B)**; 9-final product after one month ripening **(B)**; 10-cheese curd before heating **(C)**; 11-cheese curd after heating **(C)**; 12-final product **(C)**; 13-final product after one month ripening **(C)**; 14-cheese curd before heating **(D)**; 15-cheese curd after heating **(D)**; 16-final product (smoked cheese) **(D)**; 17-final product after one month ripening **(D)**.

## 4.4 Microbiological analysis

Microflora of pasta filata cheese consist of diverse bacteria, yeasts and sometimes even molds which can originate from initial microflora of milk, from starter cultures and from secondary contamination. Total counts of different groups of microorganisms were taken as parameters to analyze microbial contamination of pasta filata cheese from A-, B-, C-, and D-manufacturers. Aerobic mesophilic and psychrotrophic microorganisms and coliform bacteria, lactobacilli, lactic streptococci and yeasts were determined during cheese manufacturing (cheese curd before heating or after fermentation, cheese curd after heating), in final pasta filata cheese products and after storage. Results of microbial counts with statistical analysis are shown in Tables (7 – 12).

Numerous studies have characterized microbial species from pasta filata cheese [119,120], such as *Lactococcus lactis* subsp. *lactis*, subsp. *lactis* bv. *diacetylactis*, subsp. *cremoris*, *Streptococcus thermophilus*, enterococci such as *Enterococcus faecium* and *E. faecalis*, members of family *Enterobacteriaceae* such as *Escherichia coli*, *Klebsiella* sp., some yeasts such as *Debaryomyces hansenii* and *Kluyveromyces marxianus*, and various spoilage psychrophilic microflora [121]. Also thermoduric species of *Bacillus*, *Clostridium*, *Lactobacillus*, *Microbacterium*, *Micrococcus* and *Streptococcus* can survive milk heat treatment and grow in cheese products. The spoilage bacteria of importance in soft cheeses include psychrotrophic Gram-negative rods such as *Pseudomonas*, *Alcaligenes*, *Achromobacter* and *Flavobacterium*, which can produce odors and flavors owing to presence of lipolytic and proteolytic enzymes [122].

### 4.4.1 Microflora changes during cheese manufacturing

The first step in pasta filata manufacturing is coagulation of pasteurized cow milk. Pasteurizaton at 72°C for 15 sec kills about 99% of the bacteria of the raw milk, however, bacterial spores are not inactivated [123]. Not only spoilage microorganisms, but also enzymes capable of producing flavor and texture are inactivated by pasteurization [124]. Coagulation proceeds by activity of starter cultures (LAB) and leads into formation of firm curd [96]. Bacteria are incorporated in the casein network, so retention of bacteria in the curd is high (85-95%). The curd grains are separated from the whey, and then it is left for continued acidification,

until a pH value of about 5.1 to 5.3 is reached [123]. Afterwards, the milled curd is shoveled into hot water (55-85°C) [96] and kneaded until it is smooth and elastic.

It is well known that heating the cheese curd in hot water along with stretching causes spatial rearrangement of the protein matrix in a lattice of parallel fibers between which the fat and whey droplets lie [96]. Obviously, these processes have substantial effect on present microorganisms. In this work it was observed that heating and stretching have the most inhibition effect on coliform bacteria (if they were present). They are the most sensitive to heat and can be reduced more than 4 log CFU.g$^{-1}$ (Table 7). Psychrotrophic bacteria (Table 8) and yeasts (Table 10) were also significantly influenced – they were declined by 4 log CFU.g$^{-1}$. Mesophilic bacteria were affected at least (reduction about 2 log CFU.g$^{-1}$) (Table 9), LAB - lactobacilli (Table 11) and lactic streptococci (Table 12) remained almost untouched by the effect of high temperature and stretching process.

On the other hand, total counts of microorganism except coliforms present in the stretched curd rests high – about 4 log CFU.g$^{-1}$ for aerobic psychrotrophic and mesophilic bacteria, from almost 3 to more than 5 log CFU.g$^{-1}$ for lactobacilli and lactic streptococci, and from zero to almost 4 log CFU.g$^{-1}$ for yeasts. It should be noticed that the amount of microorganisms depends on the type of cheese in case of yeasts, lactobacilli, and lactic streptococci. According to Pluta *et al*. [125], in the grated Mozzarella cheese (Polish type) the total count of microorganisms was 9.67 log CFU.g$^{-1}$, what is two times higher than in present study, however, they have detected coliform bacteria and moulds in 0.1 g of cheese. Aponte *et al*. [126], concluded that the stretching procedure did not seem to affect LAB during manufacturing and ripening of Provolone del Monaco cheese (an artisanal pasta filata cheese).

The highest population levels were detected from the end of curd fermentation up to the salting of the cheese, with counts ranging from about 7 log CFU.g$^{-1}$ to 9 log CFU.g$^{-1}$. In other study was also observed that *Streptococcus thermophilus* and *Streptococcus macedonicus* prevailed during cheese manufacturing and survived during 9 months of ripening, together with enterococci and lactobacilli of the *casei* group, especially *Lactobacillus rhamnosus* [126].

After heating and stretching phase, the curd is extruded into a mold and cooled. According to type of cheese, dry salt or brine solution can be added after heating or the molded cheese can be immersed in salted brine.

**Table 7.** Coliform bacteria in four types of pasta filata cheese.

| | Total viable counts (log CFU.g$^{-1}$ cheese, mean±standard deviation) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Technological steps | | | Storage time | | | | | |
| | 1 | 2 | 3 | 1w | 2w | 3w | 1m | 2m | 3m |
| A | $4.53\pm0.84_{a}^{A,E}$ | ND | $1.84\pm1.74_{a,b}^{C,D}$ | $3.48\pm0.21^{A,D}$ | $5.26\pm0.18^{E}$ | $5.29\pm0.20^{E}$ | N | N | N |
| B | $2.45\pm0.44_{b}^{A}$ | ND | $2.39\pm2.98_{a,b}^{A}$ | N | N | N | N | N | N |
| C | $5.68\pm1.15_{c}^{A}$ | $1.97\pm1.84_{b}^{B,C,D}$ | $2.33\pm0.23_{a}^{B}$ | N | N | N | $1.64\pm0.25^{C}$ | ND | ND |
| D | ND | ND | ND | N | N | N | N | N | N |

Values within a column (four types of cheese) with the same superscript letter do not differ significantly ($P \geq 0.05$). Values within a line (technological steps, storage time) with the same capital letter do not differ significantly ($P \geq 0.05$).

1 – before heating, 2 – after heating and stretching, 3 – final cooled product, w-weeks, m-months, N-not tested, ND-not detected.

**Table 8.** Aerobic psychrotrophic bacteria in four types of pasta filata cheese.

| | Total viable counts (log CFU.g$^{-1}$ cheese, mean±standard deviation) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Technological steps | | | Storage time | | | | | |
| | 1 | 2 | 3 | 1w | 2w | 3w | 1m | 2m | 3m |
| A | $4.95\pm1.37_{a}^{A}$ | $4.02\pm0.50_{a}^{B}$ | $4.24\pm0.39_{a}^{B}$ | $6.84\pm0.08^{C}$ | $7.63\pm0.36^{D}$ | $9.09\pm0.15^{E}$ | N | N | N |
| B | $3.88\pm0.15_{b}^{A}$ | $3.40\pm0.51_{a}^{A}$ | $3.68\pm0.30_{b}^{A}$ | N | N | N | N | N | N |
| C | $8.26\pm0.43_{c}^{A}$ | $4.20\pm0.64_{a}^{B}$ | $5.24\pm0.27_{c}^{C}$ | N | N | N | $6.23\pm0.10^{D}$ | $5.26\pm0.10^{C}$ | $5.19\pm0.09^{C}$ |
| D | $5.80\pm0.37_{a}^{A}$ | $3.75\pm0.46_{a}^{B}$ | $4.61\pm0.12_{a}^{C}$ | N | N | N | N | N | N |

Values within a column (four types of cheese) with the same superscript letter do not differ significantly ($P \geq 0.05$).
Values within a line (technological steps, storage time) with the same capital letter do not differ significantly ($P \geq 0.05$).

1 – before heating, 2 – after heating and stretching, 3 – final cooled product, w-weeks, m-months, N-not tested.

**Table 9.** Aerobic mesophilic bacteria in four types of pasta filata cheese.

| | Total viable counts (log CFU.g$^{-1}$ cheese, mean±standard deviation) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Technological steps | | | Storage time | | | | | |
| | 1 | 2 | 3 | 1w | 2w | 3w | 1m | 2m | 3m |
| A | $5.79\pm0.75_{a}^{A}$ | $5.12\pm1.03_{a}^{A}$ | $6.70\pm0.38_{a}^{B}$ | $7.27\pm0.14^{C}$ | $7.93\pm0.15^{D}$ | $8.33\pm0.15^{E}$ | N | N | N |
| B | $3.82\pm0.14_{b}^{A}$ | $4.07\pm0.55_{b}^{A}$ | $4.83\pm1.06_{b,c}^{B}$ | N | N | N | N | N | N |
| C | $6.73\pm1.98_{a,c}^{A}$ | $4.21\pm0.65_{b}^{B}$ | $4.66\pm0.42_{b}^{C}$ | N | N | N | $5.18\pm0.15^{A}$ | $5.34\pm0.16^{A}$ | $6.01\pm0.27^{D}$ |
| D | $4.11\pm0.19_{c}^{A}$ | $3.78\pm0.16_{b}^{A}$ | $4.01\pm0.39_{c}^{A}$ | N | N | N | N | N | N |

Values within a column (four types of cheese) with the same superscript letter do not differ significantly ($P \geq 0.05$). Values within a line (technological steps, storage time) with the same capital letter do not differ significantly ($P \geq 0.05$).

1 – before heating, 2 – after heating and stretching, 3 – final cooled product, w-weeks, m-months, N-not tested.

**Table 10.** Yeasts in four types of pasta filata cheese.

| | Total viable counts (log CFU.g$^{-1}$ cheese, mean±standard deviation) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Technological steps | | | Storage time | | | | | |
| | 1 | 2 | 3 | 1w | 2w | 3w | 1m | 2m | 3m |
| A | $3.18\pm0.54_{a}^{A,D}$ | ND | ND | $2.64\pm0.09^{A}$ | $4.66\pm0.69^{C}$ | $3.88\pm0.33^{C,D}$ | N | N | N |
| B | $2.15\pm0.04_{b}^{A}$ | $2.34\pm0.10_{b}^{B}$ | $1.64\pm0.16_{b}^{C}$ | N | N | N | N | N | N |
| C | $5.96\pm0.68_{c}^{A}$ | $3.95\pm0.10_{c}^{B}$ | $3.93\pm0.26_{c}^{B}$ | N | N | N | $3.80\pm0.42^{B}$ | $4.56\pm0.09^{C}$ | $4.70\pm0.26^{C}$ |
| D | $5.21\pm0.38_{c}^{A}$ | $3.10\pm0.19_{d}^{B}$ | $4.56\pm0.20_{d}^{C}$ | N | N | N | N | N | N |

Values within a column (four types of cheese) with the same superscript letter do not differ significantly ($P \geq 0.05$). Values within a line (technological steps, storage time) with the same capital letter do not differ significantly ($P \geq 0.05$).

1 – before heating, 2 – after heating and stretching, 3 – final cooled product, w-weeks, m-months, N-not tested, ND-not detected.

**Table 11.** Lactobacilli in four types of pasta filata cheese.

| | Total viable counts (log CFU.g$^{-1}$ cheese, mean±standard deviation) | | |
|---|---|---|---|
| | Technological steps | | |
| | 1 | 2 | 3 |
| A | $3.53\pm1.20_{a,b}{}^{A}$ | $3.56\pm1.12_{a,c}{}^{A}$ | $3.58\pm1.10_{a,b}{}^{A}$ |
| B | $3.51\pm0.31_{a}{}^{A}$ | $3.57\pm0.29_{a}{}^{A}$ | $3.25\pm0.07_{a}{}^{A}$ |
| C | $4.29\pm0.99_{a}{}^{A}$ | $4.79\pm0.49_{b}{}^{A}$ | $4.71\pm0.61_{b}{}^{A}$ |
| D | $2.86\pm0.29_{b}{}^{A,B}$ | $2.51\pm0.11_{c}{}^{A}$ | $3.04\pm0.20_{a}{}^{B}$ |

Values within a column (four types of cheese) with the same superscript letter do not differ significantly (P≥0.05). Values within a line (technological steps) with the same capital letter do not differ significantly (P≥0.05).
N-not tested, 1-before heating, 2-after heating and stretching, 3-final cooled product.

**Table 12.** Lactic streptococci in four types of pasta filata cheese.

| | Total viable counts (log CFU.g$^{-1}$ cheese, mean±standard deviation) | | |
|---|---|---|---|
| | Technological steps | | |
| | 1 | 2 | 3 |
| A | $4.90\pm2.07_{a,b,c}{}^{A}$ | $4.83\pm1.45_{a,c}{}^{A}$ | $5.57\pm2.00_{a,b}{}^{A}$ |
| B | $3.12\pm0.16_{a}{}^{A}$ | $3.26\pm0.10_{b}{}^{A}$ | $3.57\pm0.10_{a}{}^{B}$ |
| C | $5.00\pm0.49_{b}{}^{A}$ | $5.35\pm0.34_{a}{}^{A}$ | $4.07\pm0.22_{b}{}^{B}$ |
| D | $3.81\pm0.43_{c}{}^{A}$ | $3.87\pm0.48_{c}{}^{A}$ | $3.98\pm0.26_{a,b}{}^{A}$ |

Values within a column (four types of cheese) with the same superscript letter do not differ significantly (P≥0.05). Values within a line (technological steps) with the same capital letter do not differ significantly (P≥0.05).
1-before heating, 2- after heating and stretching, 3-final cooled product, N-not tested.

Salt plays an essential role in cheese manufacturing with its main functions taste and preservation. However, there is a risk of contamination by yeasts or molds when using brine [96]. Aged brines develop a typical salt and acid tolerant microflora (4 to 6 log CFU.ml$^{-1}$), often with *Debaryomyces hansenii* and *Staphylococcus equorum* as the predominant species [127]. LABs are sensitive to salt and they are severely inhibited if the cheese is salted before acidification has been finished [123].

It could be expected, that extrusion, cooling and salting processes have significantly negative influence on cheese microflora. Surprisingly, coliforms, psychrotrophic and mesophilic bacteria and lactobacilli remained at the same levels or were even significantly higher than after stretching. It was observed that the content of yeasts was influenced depending on the cheese manufacturer (cheese type). Namely, it the decreased in the B-type (Mozzarella), increased in the D-type (smoked cheese), and retained the level in the C-type (salted cheese). The yeasts were no in the A-type. Since lactic streptococci are salt sensitive the counts of them, as it was expected, has significantly decreased in the C-type cheese, which is salted up to 3.2%. In other types (A, C) the amount of streptococci remain the same and only at the B type the slight increase was noticed. Placing cheeses in brines with high NaCl concentrations inactivates some bacteria, selects for others, and possibly adds some microorganisms to the cheese [124].

### 4.4.2 Microflora changes during storage

During storage of cheese water is lost and complex biochemical reactions take place as a result of interaction of the coagulant, indigenous milk enzymes, starter bacteria and secondary microflora and their enzymes [124]. Glycolytic, proteolytic, and lipolytic activities are the primary events during cheese ripening. The extent of protein and fat degradation is determined by the moisture, pH, and salinity of the cheese. Enzymes of various sources result in the production of peptides, amino acids, fatty acids, carbonyl components, and sulfur compounds [127].

Some lipases of psychrotrofic microflora, which are heat-stable and survive pasteurization, can absorb on the surface of fat globules and can act in the cheese environment [128]. Psychrotrophic bacteria such as *Pseudomonas fluorescens* cause bitter taste and bad odor through lipolytic and proteolytic reactions resulting in

spoilage of cheese [128]. Hasan *et al*. [129] also reported that psychrotrophic bacteria cause a significant spoilage problem in refrigerated dairy products due to secretion of hydrolytic enzymes especially lipases and proteases. During cheese ripening (9 months) bacterial counts progressively decreased, reaching a range of 105–106 CFU.g$^{-1}$ in the cheese ready to be consumed. *Streptococcus thermophilus* and *Streptococcus macedonicus* prevailed during cheese manufacture and survived along 9 months of ripening, together with enterococci and lactobacilli of the casei group, especially *Lactobacillus rhamnosus* [126].

No subsequent growth of coliform bacteria was observed after two month of storage of the C-type cheese. However, during storage time from one to three weeks of A-type cheese, the coliform bacteria count was increased from 3.48 log CFU.g$^{-1}$ to 5.29 log CFU.g$^{-1}$, respectively (Table 7). The aerobic psychrotrophic count of A-type cheese has exhibited increasing trend during three weeks of storage (Table 8). The psychrotrophic bacteria count increased from 6.84 log CFU.g$^{-1}$ at first week to 9.09 log CFU.g$^{-1}$ after three weeks of storage. The growth of aerobic psychrotrophic bacteria was retarded with 6 log CFU.g$^{-1}$ in the C-type cheese during three months. The significant growth of about one log cycle of aerobic mesophilic bacteria (Table 9) was noted in the A-type cheese stored for three weeks. After three months of storage of C-type cheese, the mesophilic bacteria count was expanded from 5.18 log CFU.g$^{-1}$ at first month to 6.01 log CFU.g$^{-1}$ at third month. The value of yeasts (Table 10) of the C type cheese was enhanced from 3.80 log CFU.g$^{-1}$ to 4.70 log CFU.g$^{-1}$ during three months. In case of the A type cheese stored for two weeks yeasts counts were maximum 4.66 log CFU.g$^{-1}$ and dropped to 3.88 log CFU.g$^{-1}$ after three weeks.

### 4.4.3 Biogenic amines analysis

Small amounts of some biogenic amine can usually be found in foods, because they play a natural role in animal metabolism [130]. Biogenic amines are often found in cheese but present little hazard. Nevertheless, high levels of tyramine and histamine can cause adverse health effects in mono- and diamine oxidase deficient persons. According to reports, the amount of tyramine and histamine 40-100 mg·kg$^{-1}$ causes mild poisoning and as toxic level is taken more than 100 mg·kg$^{-1}$ [103, 131, 132]. Tyramine and histamine are formed during cheese ripening from enzymatic decarboxylation of parent amino acids. However, the formation of BAs is influenced by temperature, oxygen supply and pH and these favourable conditions occur especially during spoilage of food [122]. Causative agents are often *mesophilic*

*lactobacilli* and *Enterobacteriaceae.* However, precursors (tyrosine, histidine) are present only in ripened cheese in sufficient quantities to allow for significant amine build up [131].

Analysis of BAs was performed as addition evaluation of the samples. The biogenic amines of the analyzed cheese samples are summarized in Table 13. Differences of biogenic amines were observed depending on the types of pasta filata cheese. Cadaverine, putrescine, tyramine, spermine, histamine, spermidine were found in the samples. Agmatine and 2-phenylethylamine were not detected. Cadaverine was the main biogenic amine in all samples tested. Spermine was the second. In samples of final product from A-manufacturer the content of cadaverine was significantly ($P<0.05$) higher when compared with samples from B- and D-manufacturers; but the content of this amine was significantly ($P<0.05$) lower in the samples from C-manufacturer. The median values of cadaverine was low in the samples $B_f$ and $D_f$: 2.9 $mg \cdot kg^{-1}$ and 5.2 $mg \cdot kg^{-1}$ respectively; while, much more higher content was in the samples $A_f$ and $C_f$: 28.1 $mg \cdot kg^{-1}$ and 36.2 $mg \cdot kg^{-1}$ respectively. The low median value of spermine with 0.7 $mg \cdot kg^{-1}$ is found in the sample $B_f$ in comparison to other samples. Histamine, spermidine were detected in all samples, but always in very low levels, no higher than ~6 $mg \cdot kg^{-1}$.

**Table 13.** Biogenic amines concentration in pasta filata cheese samples.

| Concentration of biogenic amines ($mg \cdot kg^{-1}$) | | | | |
|---|---|---|---|---|
| Biogenic amine | Mean±SD | | | |
| | $A_f$ | $B_f$ | $C_f$ | $D_f$ |
| cadaverine | 28.1±1.4 | 2.9±0.3 | 36.2±4.4 | 5.2±0.3 |
| putrescine | 14.8±0.6 | ND | 11.6±0.03 | 34.9±0.2 |
| tyramine | 6.8±0.4 | ND | ND | 25.2±1.2 |
| histamine | 1.4±0.1 | 3.2±0.1 | 4.4±0.7 | 2.1±0.1 |
| spermidine | 5.7±0.4 | 0.3±0.01 | 1.2±0.2 | 2.5±0.4 |
| spermine | 5.3±0.2 | 0.7±0.1 | 5.3±0.4 | 8.5±0.2 |
| agmatine | ND | ND | ND | ND |
| 2-phenylethylamine | ND | ND | ND | ND |
| **Total** | **62.1** | **13.2** | **58.7** | **78.4** |

SD-standard deviation, ND-not detected

Besides, a wide variability was noticed in distribution of tyramine and putrescine. The level of tyramine ranged from nondetected to 25.2 $mg \cdot kg^{-1}$. Statistical differences were observed for putrescine also. The amine content was substantially higher ($P<0.05$) in samples from D-manufacturer in comparison with A- and C-manufacturers. The median level of putrescine varied between nondetected to 34.9 $mg \cdot kg^{-1}$. On the whole, no statistical differences ($P \geq 0.05$) between tyramine, spermine, spermidine contents in the analyzed samples from four manufacturer was determined.

The total content of biogenic amines varied from 13.2 to 78.4 $mg \cdot kg^{-1}$. Smoked pasta filata cheese ($D_f$) showed the highest content of biogenic amines. Meanwhile, the sample $B_f$ showed the lowest total content of biogenic amines.

The production of biogenic amines is an extremely complex phenomenon, depending on several variables such as raw materials, processing conditions, growth kinetics of microorganisms, and their proteolytic and decarboxylase activities, which interact with each other [130, 133, 134]. Therefore, this observed remarkable differences and variability of biogenic amines within the pasta filata cheese types could be attributed to the specific conditions of their manufacturing. The obligatory use of pasteurized milk and the absence of long ripening explain the low biogenic amine contents found in analyzed samples. The presence of high quantities of cadaverine in cheese should be considered as a consequence of a poor hygienic quality of milk[135]. Putrescine is a natural part of milk and can move into the final product [136]. During cheese production pasteurized milk was used. In such processed milk microorganisms of *Enterobacteriaceae* family can occur. This group of microorganisms can produce biogenic amines and therefore can be a source of detected putrescine from non-starter microorganisms [137, 138]. Spermine and spermidine are present in different quantities in all types of animal cells and not formed by activity of microorganisms [139]. The observed tyramine in the analyzed samples can occur due to the increased content of contaminating microorganisms with a positive tyrosine-decarboxylase activity [140]. It is well-known, that the large amounts of biogenic amines in ripened cheese are much higher and show much more variability than in unripened cheese [130] or in cheese with a short ripening period, such as pasta filata cheeses.

### 4.5 Sensory analysis

The most informative method for the sensory analysis was the ordinal preference test. As an additional evaluation criterion descriptive sensory profiles of consistency, appetite, smell and flavor were selected. Results of the preference test are an indicator of sample popularity and can be used by manufactures for further planning.

Sensory evaluation was carried out by twelve trained panel of assessors. The role of individual assessors was to sort samples from most preferred (preference ranking scale 1) to least preferred (preference order to 6) based on their personal preference. The results of ordinal preference test, including the total count of all samples are given in Table 14.

**Table 14.** Sensory evaluation of the selected samples.

| Identification of the sample (sample code) | **A1** | **B2** | **C3** | **D4** | **E5** | **F6** |
|---|---|---|---|---|---|---|
| Assessors 1 -12 | Samples order 1 - 6 | | | | | |
| 1 | 6 | 5 | 3 | 1 | 4 | 2 |
| 2 | 5 | 6 | 2 | 1 | 4 | 3 |
| 3 | 6 | 5 | 4 | 3 | 2 | 1 |
| 4 | 4 | 5 | 2 | 1 | 6 | 3 |
| 5 | 5 | 6 | 3 | 2 | 4 | 1 |
| 6 | 4 | 5 | 3 | 1 | 2 | 6 |
| 7 | 6 | 5 | 4 | 1 | 3 | 2 |
| 8 | 5 | 6 | 3 | 2 | 4 | 1 |
| 9 | 4 | 6 | 3 | 5 | 2 | 1 |
| 10 | 5 | 6 | 4 | 1 | 3 | 2 |
| 11 | 6 | 5 | 3 | 2 | 4 | 1 |
| 12 | 6 | 5 | 3 | 1 | 4 | 2 |
| **Total** | **62** | **65** | **37** | **21** | **42** | **25** |

The best rating in the ordinal preference test was obtained for the sample D4 (the total order of 21), which is Mozzarella sample of 30 day ripening. The sample F9 (smoked cheese) was on the second place (the total order of 25). Rather poor rating was acquired for samples A1 and B2, fresh Mozzarella cheese samples from two different manufacturers-one day after manufacturing. Results of statistical evaluation using the Friedman test on 95% confidence level ($\alpha = 0.05$) showed

significant difference (P<0.05) between D4, A1, and B2 samples, and between samples F6, A1 and B2.

Results of sensory evaluation have shown a qualitative difference between ripened samples, such as Mozzarella D4 sample of 30 day ripening, the smoked cheese F6 sample of 22 days ripening, and sample of fresh Mozzarella A1 one day after manufacturing.

The distinct and characteristic flavor is acquired during cheese ripening and this fact ultimately confirmed by the results of our evaluation. The results of statistical analysis are presented in Table 15.

**Table 15.** Statistical evaluation of the sensory analysis

| | **A 1** | **B2** | **C3** | **D4** | **E5** |
|---|---|---|---|---|---|
| **B2** | N | | | | |
| **C3** | N | R | | | |
| **D4** | R | R | N | | |
| **E5** | N | N | N | N | |
| **F6** | R | R | N | N | N |

"R" -between samples there are statistically significant differences (P <0.05)
"N"-between samples there are no statistically significant differences (P ≥ 0.05)

The following text is a sensory evaluation with verbal expression and commentary for each cheese samples:

**Sample A1 - Mozzarella I, one day after manufacturing:**

- Consistency - elastic, fibrous. Color chalk-white;
- Appetite and smell - clean, milky but inexpressive, slightly acidulous;

**Sample B2 - Mozzarella II, one day after manufacturing:**

- Consistency - tougher, rubbery, fibrous. Color - greyish white;
- Appetite and smell - flavourless, insipid, clotted, weakly acid;

**Sample C3 – Mozzarella I, seven days after manufacturing:**

- Consistency-mildly plastic, soft, viscous/gummy, slightly spreadable. Color – creamy with a yellow tint;
- Appetite and smell – clean, milky, slightly acidulous, flavour – mildly typical for ripened cheeses;

**Sample D4 – Mozzarella II, 30 days after manufacturing:**

- Consistency - plastic, short to moderately spreadable, weaker fibrous structure;
- Appetite and smell – clean, milky, slightly acidulous, harmonic, fuller, typical of mature cheese;

**Sample E5 – Salted cheese, one day after manufacturing:**

- Consistency - firm, fibrous structure, slightly deliquescent. Color- slightly cream-coloured;
- Appetite and smell – predominates strongly acidic, salty appetite. Typical flavor of mature cheese is suppressed of salt appetite;

**Sample F6 – Smoked cheese, 22 days after manufacturing:**

- Consistency – shorter, slightly stiffer, elastic, slightly but spreadable. However, consistency was not homogeneous in all mass of the cheese-more ripened in the middle of cheese, due to surface layers with fibrous character. Consistency of surface layers was influenced by smoking, due to partial inactivation of microorganisms and enzymes, and, consequently, the slower proteolysis;
- Appetite and smell – more pronounced after smoking, slightly sour milk.

The performed evaluation showed that assessors preferred more mature cheese or cheese prepared by smoking. Pasta filata cheese with a high salt content are characterized by long time of shelf life, however, are less appropriate for the practical use in gastronomy.

## 5. CONTRIBUTION TO SCIENCE AND PRACTICE

The current work provides data on distribution of casein molar fractions in pasta filata cheeses. The complex of conducted analyses has shown that casein complex is relatively thermostable, i.e. under steaming standard temperature used for this technology the denaturation and degradation of the casein complex was within the tolerance.

The chromatographic as well as electrophoretic methods are applicable and meaningful for studies on changes in the casein complex.

The results of microbiological analyses showed that the inactivation of culture microorganisms during heating process is within permitted limit.

The results of BA analysis represent the fact that the amine concentration depends on the type of cheese, the ripening time. The large amounts of biogenic amines in ripened cheese are much higher and show much more variability than in unripened cheese or in cheese with a short ripening period, such as pasta filata cheeses.

Further, this study will enhance the theoretical and practical knowledge in the field of cheese production and food chemistry.

## 6. CONCLUSIONS

This PhD study is focused on determination of casein fractions distribution in Pasta filata cheeses. The results of current work have the theoretical and practical interests.

The data presented contain new information on the characterization of protein profiles of pasta filata cheeses. Electrophoretic and chromatographic methods are suitable analytical systems for the separation and analysis of protein profiles. Nevertheless, both methods are characterized by distinct results. The individual molecular weights of the samples were obtained by electrophoretic analysis. The chromatography method was used to measure changes in the distribution of number average and weight average molecular weights.

Generally, it can be concluded that SDS-PAGE clearly showed changes in the protein profiles of the samples, which occurred during the production processing and ripening of the sampled cheeses.

Electrophoretic patterns of samples from A-manufacturer were characterized by the presence of protein fractions with molecular weights of 27.0-34.6 kDa. The sample of the final product after one month of ripening of A-manufacturer was characterized by the presence of distinctive bands with low molecular weights ranging from 12 to 25 kDa. Yet electrophoretic profiles of samples from B-manufacturer did not differ significantly and changed only in the sample of the final product after one month of ripening, with the formation of low molecular mass fractions 15 and 25 kDa. Electrophoregram of C-manufacturer samples, the final product of which contained the highest amount of salt in comparison with other samples, showed 80 kDa bands which remained stable throughout heating process, including in the sample of final product after one month of ripening. In samples from D-manufacturer, 15-25 kDa bands formed in a sample of the final product after one month of ripening. All samples showed casein fragments with low m.w. 6.5-20 kDa. Active enzymes are gradually transforming the long chain of casein into a shorter one, as was observed in samples of final products and final products after one month of ripening, thus changing the protein profile.

It should be noted, that the results of chromatographic analysis showed an increase in the values of $M_n$ and $M_w$ in the samples of cheese curds after heating of samples from A-, B-, and D-manufacturers, and a decrease in this parameters in the

final product. However, along with that in samples of C-manufacturer, the reverse changes were observed, which occurred as a result of the reduction in the values of $M_n$ and $M_w$ in a sample of cheese curd after heating and the increase of these values in the final product. Nevertheless, additional work is needed and can be useful for completing a cull product characterization of pasta filata cheeses produced in the Czech Republic.

The analysis of the chemical parameters, microbiological, GPC as well as SDS-PAGE analyses of cheese curds and final products are necessary for the quality control of the cheese production.

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## LIST OF PUBLICATIONS

ONIPCHENKO, N.V., SMÉKAL, Z., HRABĚ, J. Mechanism degradation casein complex in steamed (pasta filata) cheeses. In: *6th Annual International Bata Conference for Ph.D. Students and Young Researchers.* Zlín, Czech Republic, 2010.

ONIPCHENKO, N.V., SMÉKAL, Z., HRABĚ, J. Researches of the mechanism degradation casein complex in steamed (pasta filata) cheeses. In: *Works of international scientific-practical conference. Innovative technologies and contemporary materials.* Kutaisi, Georgia, 2010. ISBN: 978-9941-417- 68-9.

ONIPCHENKO, N., DOLEŽALOVÁ, M., SMÉKAL Z., HUMPOLÍČEK, P., ČTVRTNÍČKOVÁ, L., JAN HRABĚ , J. Changes in protein profile of pasta-filata cheeses during thermal stretching-extruder process (submitted to *Central European Journal of Chemistry*, 2012)

ONIPCHENKO, N., DOLEŽALOVÁ, M., PROCHÁZKOVÁ, E., MARTINKOVÁ, I., HRABĚ, J. Microflora changes in pasta filata cheeses during manufacturing. (submitted to *Mlékařské listy*, 2012)

PROCHÁZKOVÁ, E., HRABĚ, J., KREJČÍ, J., ONIPCHENKO, N. The influence of relative humidity on changes in contents of primary and secondary oxidative products of fat in whole dried milk. *Mlékařské listy*. 130. p. IX – XII. ISSN: 1212 – 950 X. Accepted for publication (December 2011).

ONIPCHENKO, N., DOLEŽALOVÁ, M., MARTINKOVÁ, I., HUMPOLÍČEK, P., HRABĚ, J. Determination of microbiological safety of Czech pasta filata cheeses. (submitted to *Journal of Food Safety*, 2012)

# CURRICULUM VITAE

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## EDUCATION AND QUALIFICATION:

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| 1992 –2002 | Secondary school<br>(2002-Silver Medal from the Ministry of Education awarded for special achievements in studies) |
| 2002– 2007 | Donskoy State Agrarian University, Russia<br>Study branch: Production and preparation of agricultural produce<br>Master degree (with honors) |
| 2008- present | Tomas Bata University in Zlín<br>Faculty of Technology<br>Department of Food Technology and Microbiology, Czech Republic<br>Doctoral Study |

## PROJECTS:

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| 27.04.2006-03.07.2006 | Practical training as research student at Meat Processing and Packing Factory LLC "Ekaterinenskiy", Russia |

**PERSONAL SKILLS:**

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