Evaluation of picture forming methods compared to standard analytical methods for detection of structural changes caused by different thermal and non-thermal treatments of raw bovine milk

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Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit selbstständig und eigenhändig sowie ausschließlich unter Verwendung der aufgeführten Quellen und Hilfsmittel angefertigt habe.

Berlin, den 5. September 2013

Daniela Abel

Wer will das Lebendige erkennen und beschreiben,

Sucht erst den Geist herauszutreiben,

Dann hat er die Teile in seiner Hand,

Fehlt, leider! Nur das geistige Band!

- Johann Wolfgang von Goethe, Faust I

This thesis is dedicated to Amadeus and Paul

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Meiner Familie und ganz besonders meiner Mutter und meinem verstorbenen Vater möchte ich für die finanzielle Unterstützung während meines Studiums danken.

Abstract

Initiated by Rudolf Steiner in the early twentieth century, picture forming methods (PFMs) offer a more holistic view on food quality compared to standard analytical methods. In this thesis, standard analytical methods and the PFMs Steigbild, circular chromatography and biocrystallization were compared for their ability to detect protein denaturation and other structural changes caused by different treatments of raw bovine milk.Fresh raw milk was treated with four different processing methods of which two cause protein denaturation (heating at 75°C for 15 s and 85°C for 5 min, high hydrostatic pressure treatment [HP] at 300 and 600 MPa for 10 min) and two, which do not cause denaturation (skimming, pulsed electric fields treatment [PEF] at 45 and 116 kJ/kg). Protein analysis was carried out by means of RP-HPLC Pictures were evaluated via an online image analysis test as an adaption of the triangle test. Additionally, computerized texture and structure analysis were carried out for the biocrystallograms. With the Steigbild method it was possible to differentiate between untreated and HP treated samples. Also, heat treated and skimmed milk showed some significant results. PEF treatment gave no differentiation. With the circular chromatographic method, no treatment-induced alterations were observed, however a linear relationship between the area of lowest optical density and the protein content was detected. For both methods, reproducibility was consistently poor. Differences found in the online image analysis of the biocrystallograms were significant for HP treatment and skimming and showed good reproducibility. Structure analysis confirmed the results of the online image analysis and was able to detect changes in heat and PEF treated samples. It was shown that HP and skimming had different effects on the crystallization structure whereas the impact of heat and PEF treatment was similar. The results suggest that the PFM Steigbild and biocrystallization are sensitive towards protein denaturation in milk plus other structural changes like alterations in the fat content.

Zusammenfassung

Angeregt von Rudolf Steiner zu Beginn des letzten Jahrhunderts bieten die sogenannten bildschaffenden Methoden (PFMs) einen ganzheitlicheren Blick auf die Lebensmittelqualität als die heute üblichen Standardanalysemethoden. In dieser Diplomarbeit wurden Standardanalyse- und die bildschaffende Methoden Steigbild, Rundfilterchromatographie und Biokristallisation dahingehend verglichen, in wieweit sich Proteindenaturierung und andere strukturelle Veränderungen, hervorgerufen durch verschiedene Behandlungen von roher Kuhmilch, damit nachweisen lassen. Tagesfrische Milch wurde mit vier verschiedene Verarbeitungsmethoden behandelt. Darunter zwei, die eine Proteindenaturierung in Milch bewirken (Erwärmen bei 75°C für 15 s und bei 85°C für 5 min und Hochdruckbehandlung [HP] bei 300 MPa bzw. 600 MPa für je 10 min.) und zwei die jeweils zu anderen strukturellen Änderungen führen (entfetten und Behandlung mit Hochspannungsimpulsen [PEF] bei 45 kJ/kg und 116 kJ/kg). Jedes Experiment wurde wiederholt, um die Reproduzierbarkeit zu überprüfen. Eine Analyse der Proteine wurde mittels RP-HPLC durchgeführt. Die mittels PFMs erhaltenen Bilder wurden im Zuge einer Online-Bilderanalyse, einer Adaption des Triangel-Tests, ausgewertet. Bei der Biokristallisation standen zusätzlich noch die computergestützen Textur- und Struktur-Analysetools zur Verfügung. Mit der Steigbildmethode was es möglich, zwischen den unbehandelten und den HP-behandelten Proben zu unterscheiden. Die Bilder der entfetteten und hitzebehandelten Proben zeigte ebenfalls einige sigifikante Resultate. PEF-behandelte Proben führten zu keiner Differenzierung. Bei den Rundfilterchromatogrammen wurden keine behandlungsabhängigen Änderungen detektiert, jedoch wurde ein linearer Zusammenhang zwischen der Fläche der geringsten optischen Dichte und dem Proteingehalt der Proben festgestellt. Beide Methoden wiesen sich durch eine durchweg schlechte Reproduzierbarkeit aus. Signifikante und reproduzierbare Ergebnisse wurden bei der Online-Analyse der Biokrisallogramme für HP-behandelte und entfettete Proben festgestellt, welche durch die Resultate der Strukturanalyse bestätigt wurden. Außerdem konnten strukturelle Änderungen in erhitzten und PEF-behandelten Proben nachgewiesen werden, welche sich als ähnlich erwiesen. Die Ergebnisse deuten darauf hin, dass die PFMs Steigbild und Biokristallisation empfindlich gegenüber Proteindenaturierung und anderen strukturellen Änderungen in Milch reagieren, wie zum Beispiel Veränderungen im Fettgehalt.

Contents

List of Appendices

List of Figures

List of Tables

List of Abbreviations and Symbols

Abbreviations

Abbreviation	Meaning
UT	Untreated samples
ANOVA	Analysis of variance
BC	Biocrystallization
BCS	Binary crystallization structure
BSA	Bovine serum albumin
Comp.	Component
DAD	Diode array detector
DC	Direct current
GLCM	Greylevel Co-occurrence Matrix
H	Heat treated samples
HMF	5-Hydroxymethylfurfural
HP	High hydrostatic pressure
HPLC	High-performance liquid chromatography
HTST	High temperature short time
HV	High voltage
IgM	Immunglobulin M
ISI	Innovative steam injection
LA	Lactalbumin
LG	Lactoglobulin
LME	Linear mixed effects (model)
NPC	Normal phase chromatography
PCA	Principal Component Analysis
PE	Polyethylene
PEF	Pulsed electric fields
PFM	Picture forming method
RGB	Red-green-blue (colour space)
ROI	Region of interest
RP-HPLC	Reversed-phase high-performance liquid chromatography
RPC	Reversed phase chromatography
tBME	tert-Butyl methy lether
TFA	Trifluoroacetic acid

UHT	Ultra high temperature
WP	Whey proteins
Z	Centrifuged samples

Greek Symbols

Symbol	Meaning	Unit
η	Viscosity	$N \cdot s \cdot m^{-2}$
ν	Separation speed	$m \cdot s^{-1}$
ω	Angular velocity	s^{-1}
ρ	Density	$kg \cdot m^3$
ρ_a	Electrical resistivity	$\Omega \cdot m$
ρ_{fg}	Density of the milk fat globules	$kg \cdot m^3$
ρ_{pl}	Density of the milk plasma	$kg \cdot m^3$
σ	Conductivity of the medium	$S \cdot m^{-1}$
τ	Pulse width	$\mu \cdot s$
τ_h	Holding time	s

Latin Symbols

Symbol	Meaning	Unit
a_Z	Centrifugal acceleration	g
C	Capacitance	F
d	Diameter	m
d_i	Interelectrode gap	m
$E_{(crit.)}$	(Critical) Electric field strength	$kV \cdot cm^{-1}$
f	Frequency	s^{-1}
F_R	Radial force	N
g	Gravitational acceleration	g
l	Length of the tube	m
m	Mass	kg
$\dot{m}$	Mass flow rate	m^3/s
n	Number of pulses	
p	Pressure	MPa
p	Probability	
Q	Energy stored by the capacitor	J
R	Radius	m
r	Radius	m
$\overline{R}^2$	Adjusted coefficient of determination	

R_a	Resistance of the treatment chamber	Ω
S	Size of the electrode surface	m^2
T	Temperature	°C or K
t	Treatment time	s
T_i	Samples treated with intensity i	
t'_{Ri}	Net retention time of component i	min
t_0	Dead time	min
t_{Ri}	Gross retention time of component i	min
U	Voltage	V
$\dot{V}$	Flow rate	$l \cdot h^{-1}$
V_t	Volume of the final silicon tube	m^3
w/v	Weight-Volume percentage concentration	%
w/w	Weight-Weight percentage concentration	%
w_i	Peak base width of component i	min
W_s	Specific energy	$kJ \cdot kg^{-1}$
W_{pulse}	Energy per pulse	J
v/v	Volume-Volume percent concentration	%

1 Introduction

Processing of food has a long tradition as it enhances safety by killing potentially harmful micro-organisms, it inactivates unfavourable enzymes and leads to better digestability of some foods. Common processing methods like heating have one main disadvantage: they often lead to a high loss of sensitive nutrients, above all vitamins and the formation of unfavoured compounds. In the last decades, novel non-thermal methods, like the application of high hydrostatic pressure (HP) or pulsed electric fields (PEF) have been developed, which process the food more gently, thus nutrient losses could be minimized.

In general science, treatment-induced alterations in a (food) sample are monitored via standard analytical methods, which aim to detect changes happening to single substances in the food. In contrast to these methods, Rudolf Steiner among others introduced the so called holistic picture forming methods (PFMs) for the determination of food quality. They are called holistic, because the methods work with the whole food sample instead of detecting single compounds. Pictures with characteristic patterns are obtained. These patterns are then interpreted by trained or untrained test subjects or with relatively new computerized image analysis tools.

The supporters of these methods claim that "the whole is more than the sum of its parts" (Aristotle) and food quality cannot be solely interpreted as the sum of nutrients and the absence of hazardous substances. They argue that there must be something that "binds the food together". The famous quantum physicist Erwin Schrödinger (1944) first introduced the concept of 'order' related to food in his work *What is Life?* Today's alternative researchers (for example Balzer-Graf, 2001; Kusche et al., 2010) believe that this 'order' or 'structure' is linked with food quality and shows itself in the formation of specific patterns in the images obtained from PFMs. Hereby, processes of self-organization play a role, for example the Bénard-Maragoni-phenomenon. Processing is said to change the 'order' and thus the patterns of the images. Because it is impossible to detect this 'order' with standard analytical methods, PFMs were not recognized by general science for a long time. This only changed in the last decade with the standardization and validation of the Steigbild and biocrystallization method. PFMs might develop into an additional tool for food quality determination, next to standard analytical methods.

In this thesis the informative value of standard analytical methods (above all high-performance liquid chromatography, HPLC) is compared to the information gained by the application of PFMs. The objective hereby is to find out, whether the PFMs Steigbild, circular chromatography and biocrystallization can detect structural changes in

milk samples that are caused by different thermal (heating) and non thermal treatments (HP, PEF, skimming). For the case of milk, the modification of milk proteins (caseins and whey proteins) is a major unfavourable impact of several processing methods. Two of the chosen methods lead to protein denaturation in the milk (heat and HP) whereas the other two do not show significant effects on milk proteins (PEF, skimming). Are PFMs sensitive to the modifications of proteins in the milk and can other structural changes be detected that occur during processing? Can PFMs identify alterations that are not detectable by standard analytical methods and is thus the whole more than the sum of its parts?

2 Theoretical background

In this chapter the literature related to milk composition, treatments of milk (heating, HP and PEF treatment and skimming) as well as the standard analytical method HPLC and the PFMs (Steigbild, circular chromatography and biocrystallization) is reviewed. Particular emphasis was given to the influence of processing on milk constituents and the mode of action of the PFMs.

2.1 Milk

For thousands of years, mankind uses milk of different animals as foodstuff. Today, most milk being consumed in the world is that of cows, mainly of the Holstein breed (Belitz et al., 2008). The white liquid is mostly regarded as staple food and said to be important within the framework of a healthy diet. However, doubt about the healthiness of milk and milk products has risen in the last years. For safety reasons, milk is almost exclusively sold in heated (pasteurized or ultra-high-temperature [UHT] treated) form as harmful micro-organisms originating for example from the cow barn or the processing factory could be found in milk and be potentially harmful for humans (Krämer, 2006).

Milk is not only consumed in its original form, but also milk products, like yoghurt or cheese. In the form of powdered milk it is fed to babies as a substitute for mother's milk or used in different processed foods, such as milk chocolate. The food and pharmaceutical industry makes use of powdered milk components like proteins or lactose for 'functional food' or for pharmaceuticals. (Töpel, 2004)

2.1.1 General milk composition

Bovine milk is a very complex system of various components, such as fats, proteins, sugar (lactose), minerals, vitamins and immunoactive substances. Whereas some substances occur in dissolved form like lactose, others like the protein fraction caseins or fat exist as dispersed particles. Thus milk can be considered as an emulsion or a polydisperse system with the serum phase as a dispersion medium containing water plus all dissolved compounds and several dispersed phases such as the caseins or the fat. (Töpel, 2004)

Figure 2.1 overviews the composition of milk in matters of its nutritional value for humans (and other mammals). The substances serve two different purposes: nutrition and activation of the immune system via immunoactive substances such as

immunoglobulines among others, which is especially important for newly borns. The nutrients have several functions: supply, material for building new cells, tissue etc. (macro-nutrients) or regulation like vitamins and salts. The composition and concentration of the single compounds is not stable but dependent on several factors like breed, age, lactation period[1] and fodder. For example, the average protein concentration of milk can range between 2.8 and 4.0 % within the lactation period (Töpel, 2004; Tscheuschner, 2004). In this thesis, the main focus is on the milk proteins and the milk fat.

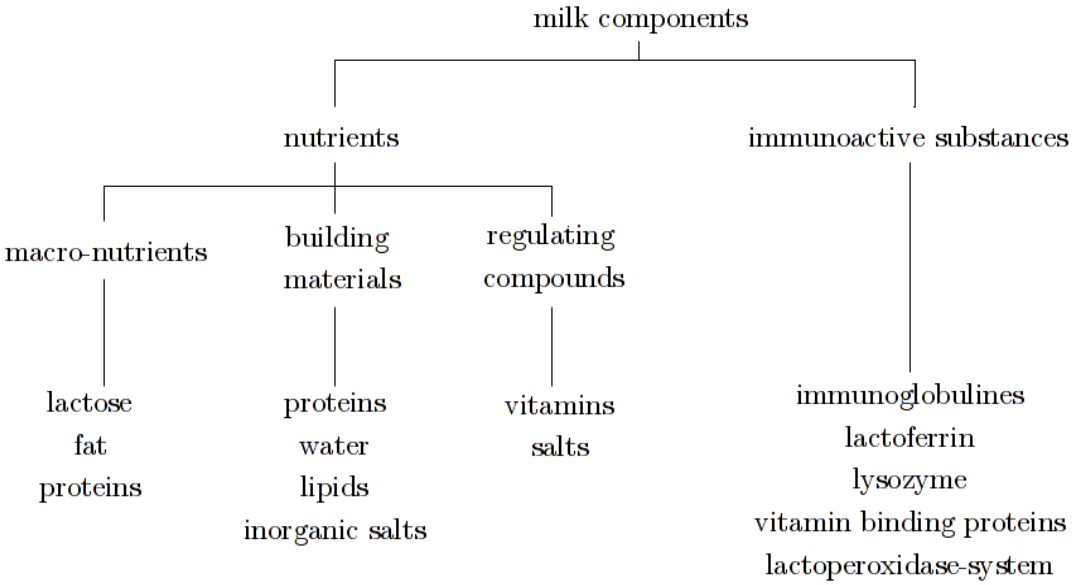


Figure 2.1: Composition of bovine milk (Töpel, 2004, Übersicht 1.1, p. 1).

2.1.2 Milk proteins

As shown in figure 2.2, one can distinguish between several different fractions: major and minor proteins. Caseins and whey proteins are the major milk proteins and represent around 98 % of total milk proteins (Töpel, 2004). Hereby, caseins account for about 76-86 % and whey proteins for about 14-24 % of total proteins in the milk (Baltes, 2007). The protein fractions that were considered in this thesis are marked in italics in figure 2.2 and will be discussed below.

In general, proteins are macro-molecules with a three-dimensional structure. They are built up out of small molecules, the amino acids that form a long string, the so called polypeptide chain or primary structure. A steric order is formed by hydrogen bonds, other non-covalent bonds or disulfide bonds among the amino acids: the so called secondary structure, for example α-helices or β-sheets. Through additional disulfide bonds, ionic bonds or van der Waals forces the proteins are folded further

[1] time between birth of the calf and the drying of the cow. The lactation period lasts around 270-300 days (Baltes, 2007).

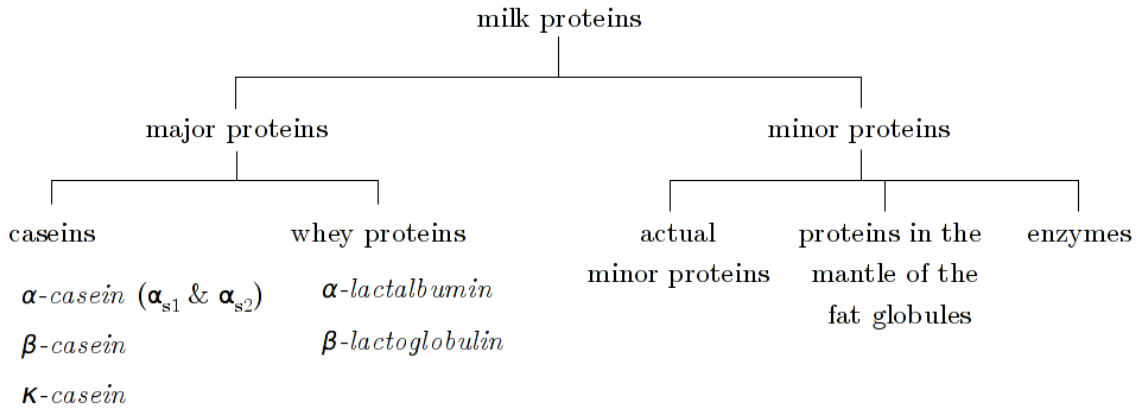


Figure 2.2: Protein composition of bovine milk. Proteins marked in italics will be discussed in this thesis (Töpel, 2004, Übersicht 7.4, p. 226).

into the tertiary structure. A quaternary structure is formed when several proteins inheriting tertiary structure interact, through disulfide bonds and non-covalent bonds. (Belitz et al., 2008)

Proteins can be distinguished by their form: there are globular proteins and fibrous proteins. Globular proteins such as whey proteins are water soluble whereas the fibrous proteins are not. (Töpel, 2004)

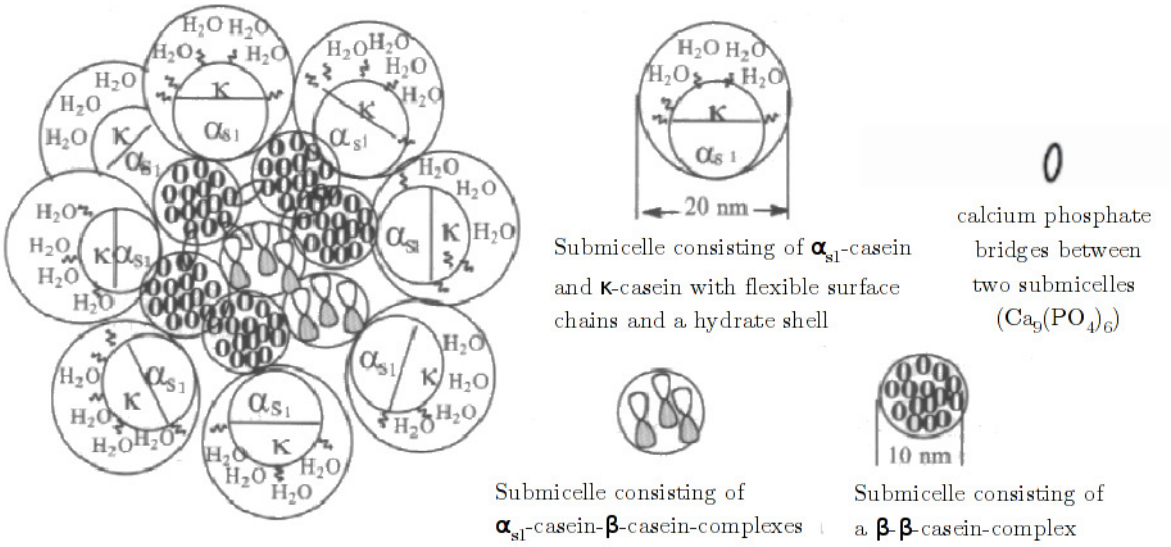


Figure 2.3: Composition of a casein micelle out of sub-micelles (Töpel, 2004, Abb. 7.28, p. 265, modified after Ono and Obata, 1989).

Caseins As mentioned above, caseins form the major part of the protein fraction in milk. There are four important casein fractions: α_{s1}-casein (34 % of total milk proteins), α_{s2}-casein (8 %), β-casein (25 %) and κ-casein (9 %) (Belitz et al., 2008). All of these casein fractions exist in different genetic variants, differing in some parts

of the primary sequence of amino acids. Here, only the major casein fractions will be regarded, whereby the α-caseins are grouped together (see figure 2.2). Caseins are phosphorous proteins and usually aggregated in micelles. The micelles are not soluble in the milk but stabilized through the other substances present in milk. There are many theories about what the casein structure looks like exactly (Phadungath, 2005). We will further focus on the model of Töpel (2004), following the model of Ono and Obata (1989) who proposed the micelle consisting of sub-micelles as shown in figure 2.3.

There are different types of sub-micelles: those, consisting of α-caseins and κ-casein, only β-casein or sub-micelles consisting of α-caseins and β-caseins, whereby the former is usually located on the surface of the micelle and the two latter can generally be found in its hydrophobic center. The calcium found in milk could potentially denature the α- and β-casein micelles. This is prohibited by the surficial κ-casein, which is very stable in the presence of calcium. Due to its amphiphile character, κ-casein stabilizes the boundary surface of the micelle. Next to κ-casein, α_{s2}-casein also shows an amphiphile character, whereas α_{s1}-casein acts hydrophilic and β-casein hydrophobic. The sub micelles are bound together by calcium phosphate bridges ($Ca_9(PO_4)_6$), see figure 2.3. (Töpel, 2004; Belitz et al., 2008)

However, caseins are prone to low pH and denature at pH 4.6[2]. The average size of the micelles ranges between 30 and 300 nm, so the micelles can (partly) be separated by ultracentrifugation. (Töpel, 2004)

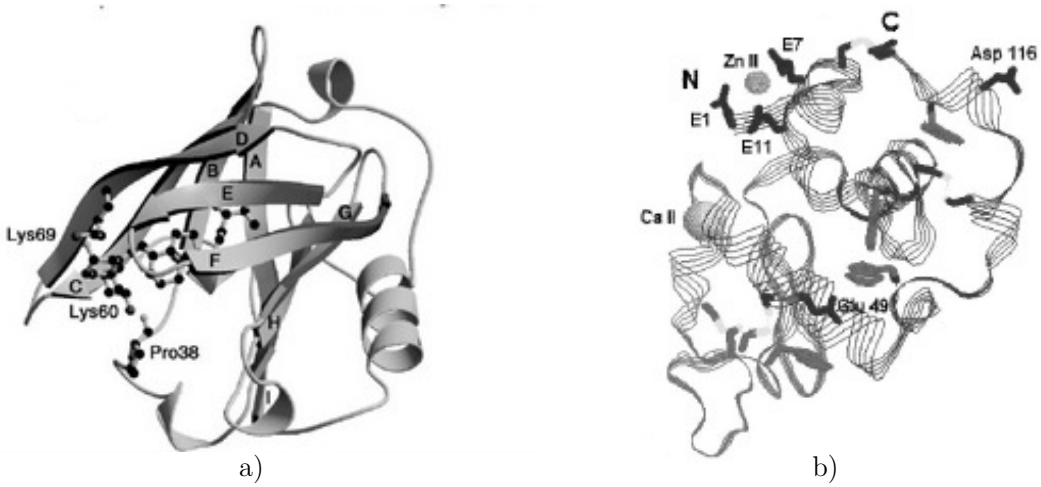
The different casein fractions show a similar molecular weight and peptide chain length ranging from 19 kDa and 169 amino acids for κ-casein and to 25.2 kDa and 207 amino acids for α_{s2}-casein (Töpel, 2004; Belitz et al., 2008).

Sensitivity to treatment conditions like high temperatures or high hydrostatic pressure will be discussed below in section 2.2.

Whey proteins Around 20 % of the total milk proteins are whey proteins, which have a globular structure. Unlike the caseins, whey proteins are soluble in the milk, even at a pH of 4.6. There exist several different whey protein fractions: α-lactalbumin (9 % of total milk proteins), β-lactoglobulin (4 %), proteose-peptone (4 %), immuno-globulin (2 %) and bovine serum albumin (1 %) (Belitz et al., 2008) from which only the former two will be regarded further in this thesis. All whey proteins exist in several genetic variants, for example β-lactoglobulin A and B, which only differ in two amino acids (Töpel, 2004).

β-lactoglobulin is strongly structured. It consists of 10-15 % α-helices, 43 % β-sheets and 47 % of orderless structures (Töpel, 2004). Dependant on the pH, the monomers have the tendency to cling together and form dimers and octamers, respectively: in the range of pH 5.5 - 7.5 it is found in the form of dimers, at pH 3.5 to 5.5, especially at pH 4.6 it exists as octamers. Below pH 3.5 and above pH 7.5 those bonds

[2]The usual pH of milk lies between 6.5 and 6.8 (Töpel, 2004).



a) b)

Figure 2.4: Structure of whey proteins: a) β-lactoglobulin with bound vitamin D_2 (Kontopidis et al., 2004, Figure 2.A), b) α-lactalbumin (Permyakov and Berliner, 2000, Fig. 1, p. 270).

collapse and the β-lactoglobulin exists solely it its monomeric form. The monomer shown in figure 2.4 a) contains five cystein tails from which one exists in a free state, hence within the folded molecule. Through (partial) denaturation, this cystein tail and with it the reactive SH-group can be uncovered, which then leads to dimerization of two β-lactoglobulin molecules or to reaction with other milk proteins, particularly κ-caseins and α-lactalbumins (Belitz et al., 2008). As shown in figure 2.4, each β-lactoglobulin monomer binds one ergocalciferol (vitamin D_2) molecule shielding it from oxidation. With 162 amino acids and a molecular weight of 18.3 kDa it is smaller than the casein fractions.

The structure of α-lactalbumin is similar to that of lysozyme found in egg white. The tertiary structure is stabilized by Ca^{2+}ions and four disulfide bridges built by eight cystein tails, compare figure 2.4. α-lactalbumin also has a biological function: it is involved in the biosynthesis of lactose as a subunit of the enzyme lactose synthase in the mammary gland (Brew et al., 1968). It shows the form of an ellipsoid and contains 26 % α-helices, 14 % β-sheets and 60 % orderless structures. With 123 amino acids and a molecular weight of 14.2 kDa it is the smallest protein fraction discussed in this thesis. α-lactalbumin is a metal protein: it binds one calcium ion per aspartic acid tail. Calcium ions are only released below pH 4. (Töpel, 2004; Belitz et al., 2008)

Sensitivity to treatment condition swill be discussed below in section 2.2.

2.1.3 Milk fat

Bovine milk contains usually between 3.0 and 4.5 % fat (Töpel, 2004). The concentration underlies natural fluctuations mentioned above and can even be higher or lower.

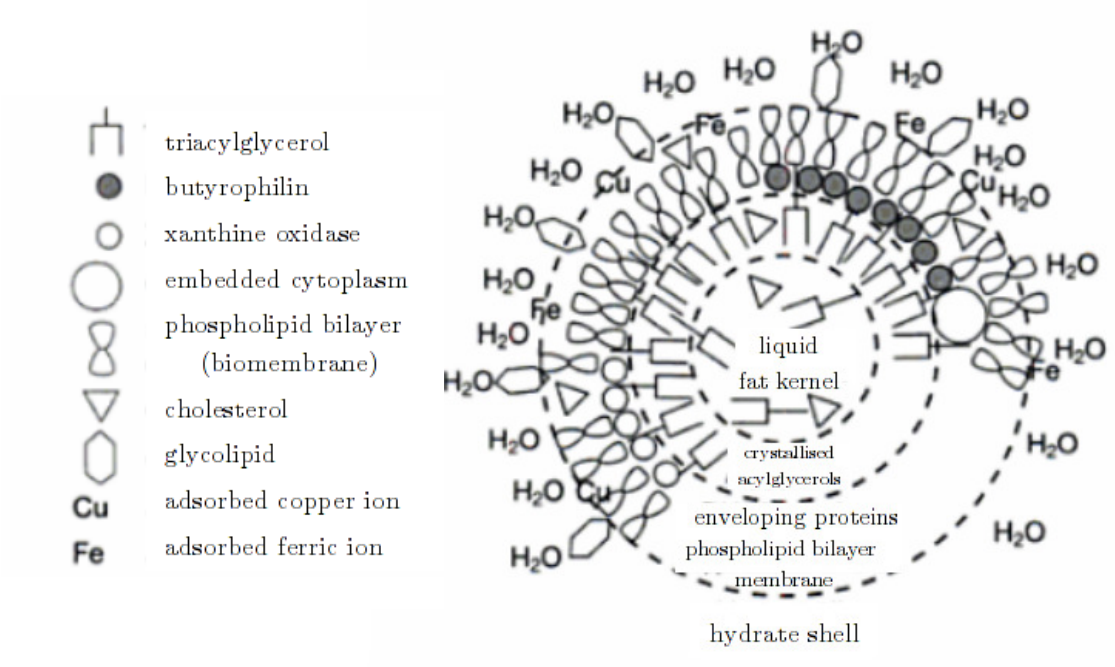


Figure 2.5: Schematic illustration of bovine fat globules with membrane (Töpel, 2004, Abb. 6.11, p. 179).

Milk fat is existent as emulsified lipid droplets with a size between 0.1 and 10 μm. The size distribution of the fat globules is subject to fluctuations within the lactation period and depending on the fodder. With a density of 0.930-0.933 g/ml at 20 °C milk fat is lighter than milk serum (ρ = 1.032-1.035 g/ml) and so the milk fat globules slowly rise to the milk's surface when left stand. The process is enhanced by lower temperatures (4-6 °C) as immunglobulin M (IgM) binds to the fat globules and clusters of fat globules are formed, which rise faster than the single droplets. This process is called creaming and can be avoided my lowering the average diameter of the fat globules via homogenization. It can also be used to skim the milk or standardize the fat content via centrifugation (Töpel, 2004; Belitz et al., 2008).

Main lipid class are triacylglycerols, counting for 98.3 % of all lipids found in milk. The rest consists of diacylglycerols (0.3 %), free fatty acids (0.1 %), phospholipids (0.8 %), sterols (0.3 %) and traces of carotenoids, fat-soluble vitamins and flavour compounds (MacGibbon and Taylor, 2006).

The composition of a bovine fat globule is shown in figure 2.5. A phospholipid layer encloses the fat kernel containing triacylglycerols and cholesterol. Next to that, the fat globule is not built up homogeneously. Enveloping proteins like butyrophilin or xanthine oxidase can be found between bilayer and fat kernel. On the side of the phospholipid layer facing the serum phase glycolipids, copper and ferric ions are stored. The glyco- and phospholipids are electrically charged and thus build up a hydrate shell around the fat globule, which keeps up the emulsified state and prevents early creaming. (Töpel, 2004)

2.2 Milk treatments

As mentioned above, milk is usually not sold raw but in processed form for safety and functional reasons. Heating, fat-standardization and homogenization of the fat globules to prevent creaming are the most common processing methods. Milk can also be processed further into milk products such as cheese, which will not be discussed here, however. Two standard milk treatments, heating and skimming and two novel treatments, high hydrostatic pressure and PEF treatment will be discussed. The treatments also differ in the degree in which they affect the milk proteins: whereby heat and hydrostatic pressure lead to protein denaturation (Cheftel and Dumay, 1996; de Jong, 2008), the two others skimming and PEF treatment do not or in marginal amounts (Sampedro et al., 2005). In the following, we will dwell on fields of application, modes of operation and the effects of the treatments on the substances in milk.

2.2.1 Heat treatment

Heating is probably the most common treatment of milk. It is applied to kill potentially harmful microorganisms and therefore also extend its shelf life while at the same time it aims to reduce negative effects of milk substances like vitamins of proteins. All heating processes represent a compromise between food safety and the adverse effects of milk substances with impacts on sensory and technological properties (Töpel, 2004).

Fields of application and modes of operation Today, there are many different approaches for the heat treatment, which are classified in the following according to de Jong (2008) if not mentioned otherwise. An overview of the general classification of heat treatments and the used equipment is shown in figure 2.6. Figure 2.7 shows the temperature-time dependency of the heat treatments discussed here.

Thermization The aim of thermization is to reduce the number of micro-organisms up to factor 10^4. Typically, the milk is treated at 68°C for 10 s or at 65°C for 20 s but has to be heated up to at least 57°C. Thermization is thus a very mild treatment, which is however not used widely when liquid milk is the end product, because inactivation of micro-organisms is too limited.

Pasteurization Under pasteurization we understand "a heat treatment aimed at reducing the number of harmful microorganisms, if present, to a level, at which they do not constitute a significant health hazard" (de Jong, 2008, p. 12). Pasteurization can be carried out in two ways: as a batch- or a continuous operation. Batch pasteurization means the milk or any other medium is heated in a closed tank whereas continuous pasteurization is usually performed in a heat exchanger followed by a holding tube for the time required. The latter usually is used when short processing times are aspired,

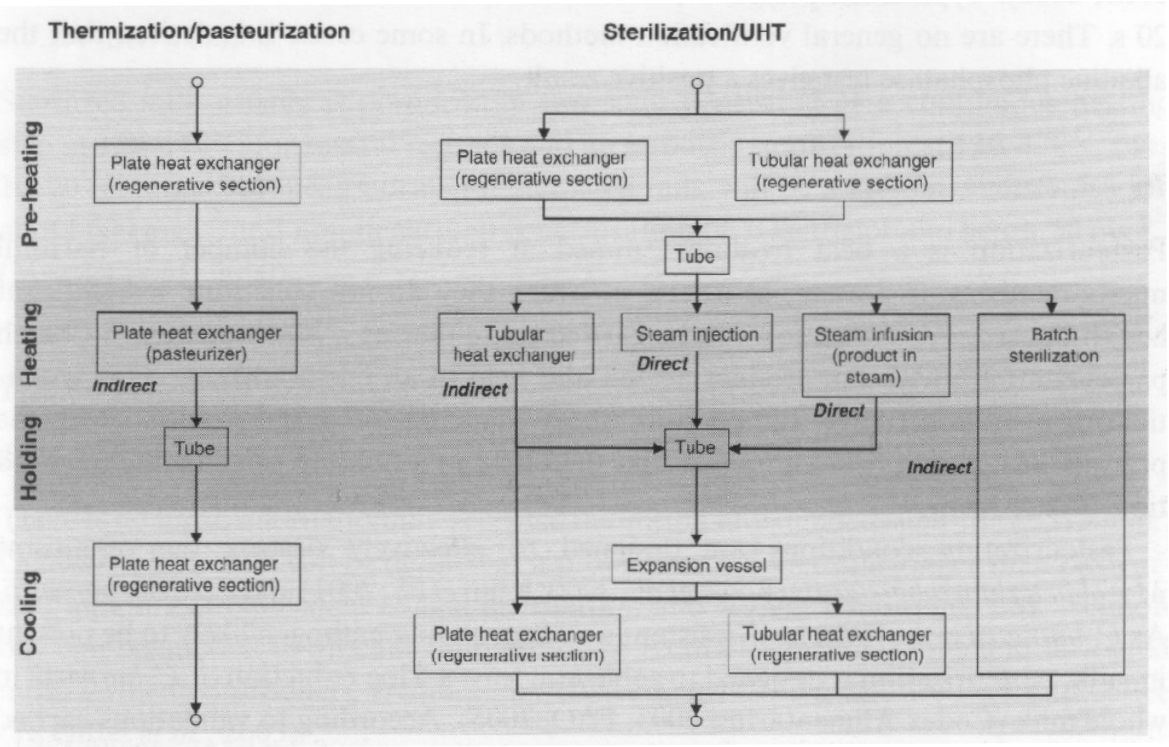


Figure 2.6: General classification of heat treatments and the type of equipment used (de Jong, 2008, fig. 1.6, p. 11).

for example as it is the case with the so called High Temperature Short Time (HTST) pasteurization. The minimum operational requirements to assure the inactivation of harmful micro-organisms are holding conditions of 75°C for 15 s in the case of HTST and 63°C for 30 min during batch operation.

UHT Ultra-high-temperature processing (UHT) of milk aims to decrease the number of the harmful bacteria *Clostridium botulinum* by twelve log-cycles. Besides that, all other viable micro-organisms and their spores are inactivated so that the milk can be stored at room temperature for several weeks. Thus, the treatment has to be more intense than pasteurization and contamination after heating has to be prevented. Usual UHT treatment conditions range between 135-150°C with a holding time of 2-4 s. UHT treatment can be performed in two ways: indirect with the aid of heat plate or tube heat exchangers or direct with hot steam injection or infusion whereby the added water is removed via flash cooling (Lewis and Heppell, 2000).

A fairly new preservation method is the innovative steam injection (ISI), which is based on the UHT treatment but is applied at very short times and even higher temperatures, usually 0.1 s at 150-200°C. The advantage of this method is the inactivation of heat-resistant spores while reducing the impact on important components (de Jong, 2008).

Sterilization Important to obtain sterile milk - meaning it's free of potentially reproductive microbes and spores - is the strict prevention of microbial contamination after the treatment, so the packaging has to be performed under aseptic conditions. This is achieved by heating the packaged milk at 110-120°C for 10-20 min after continuously heating at 120-140°C for several seconds before packaging. Sterilized milk can be stored for several years.

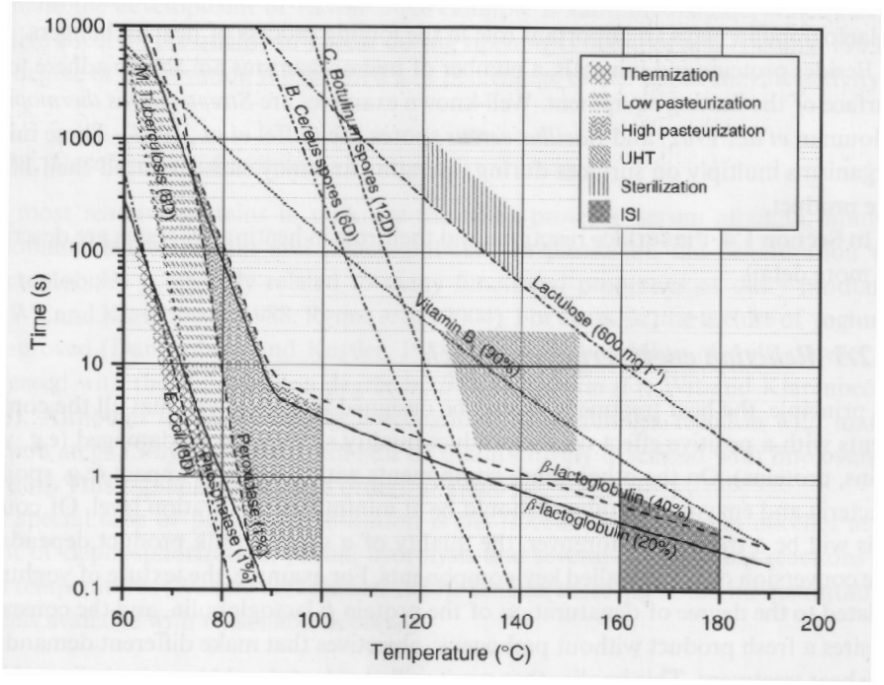


Figure 2.7: Temperature-time dependency of several heat-induced reactions (de Jong, 2008, fig. 1.2, p.8). D = decimal reduction.

Effects of heat on milk Next to the inactivation of microorganisms as a desired aim, the various heat treatments also have undesired effects on the milk substances leading to loss of nutritional value and off-flavour (Töpel, 2004). As milk is a complex system, there occur many physical, chemical and biochemical reactions. After de Jong (2008, p. 3) these reactions can be divided into five groups:

1. inactivation of micro-organisms
2. inactivation of enzymes
3. denaturation of protein
4. loss of nutrients
5. formation of new compounds

Whereas the former two are favoured types of reaction - enzymes active in milk usually have a negative effect on flavour and shelf life - the three latter effects are rather unfavourable and are discussed more detailed. The temperature-time dependencies of the

inactivation of some potentially harmful micro-organisms such as *E. coli* and bacterial spores, for example from *Bacillus cereus* are shown in figure 2.7. Generally the inactivation of bacterial spores requires higher temperatures and longer treatment times. It would be beyond the scope of this thesis to discuss the heat induced inactivation of micro-organisms in more detail. For more information see for example Lewis and Heppell (2000); Töpel (2004); Krämer (2006); de Jong (2008).

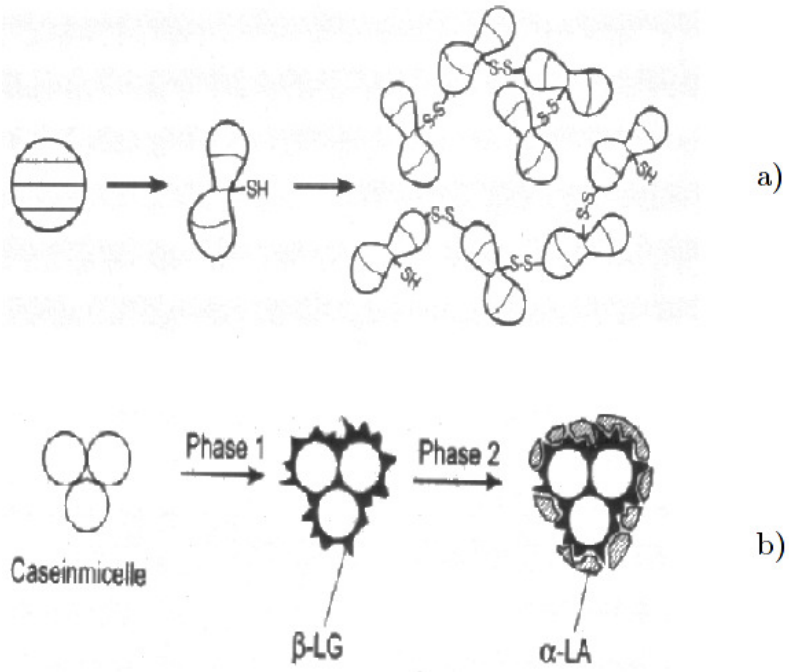


Figure 2.8: Effects of heat on milk proteins: a) model for the unfolding and aggregation of β-lactoglobulin (Hinrichs, 2000, Bild 5.1, p. 75), b) model for the modification of the casein micelle surface, explained in the text (Hinrichs, 2000, Bild 6.3, p. 105).

Heat-induced denaturation of proteins The protein fractions in milk mentioned in section 2.1 behave very differently to the increased temperature.

Casein micelles are very heat stable. They can stand 140°C for 20 min without coagulation (Töpel, 2004). However, it was observed that for heat treatments below 70°C, the casein size decreased, whereas for treatments up to 100°C the average micelle size increased or decreased depending on the pH. Above 70°C κ-caseins were found to dissociate into the serum phase (Considine et al., 2007).

Whey proteins are much more prone to heat induced denaturation than caseins and are completely denatured after 10 min heat treatment at 90°C. The denaturation of β-lactoglobulin starts at 65°C but is still reversible at this stage. At 70-75°C significant irreversible denaturation occurs, compare figure 2.7. During denaturation, the reactive SH-group mentioned above is uncovered and binds to κ-casein, see figure 2.8 b) Phase 1 or connects with other β-lactoglobulin molecules, see figure 2.8 a). This leads to the

perceived cooked taste of heated milk (Töpel, 2004). α-lactalbumin is the most heat stable whey protein. Denaturation starts at 72°C. At higher temperatures, around 80-90°C α-lactalbumin binds to the denatured β-lactoglobulin aggregated with κ-casein (see figure 2.8 b) Phase 2. At UHT conditions, β-lactoglobulin also reacts with α_{s2}-casein via disulfide bonding (Considine et al., 2007).

At high temperatures, both α-lactalbumin and β-lactoglobulin interact with the fat globule membrane forming disulfide bonds. The other whey proteins (e. g. bovine serum albumin or immunglobulin) already denature at 65°C (Töpel, 2004). Consequently, creaming in milk happens slower as the denatured IgM is not longer able to form clusters with the fat droplets. Additionally, the enveloping proteins in the fat globule also experience changes during heat treatment (Considine et al., 2007).

Heat-induced loss of nutrients According to Töpel (2004); de Jong (2008), the nutrient that is most affected by heat is thiamine (vitamin B_1). Pasteurization causes only marginal losses for the vitamins, B_1, B_2, B_6, B_{12}, C and folic acid. In the case of UHT heated milk the losses range in general between 5 % for the vitamins, B_1, B_2, B_6 and folic acid and 20 % for vitamin C and B_{12}. The biggest losses occur during sterilization: Up to 50 % of the vitamins are destroyed. The temperature-time dependency of the impacts of heat on vitamin B_1 are shown in figure 2.7. Next to the influence of the temperature, the loss of vitamins is also dependent on the presence of oxygen, the pH, light and traces of heavy metals. (Töpel, 2004)

Heat-induced formation of new compounds The heat induced formation of new compounds mainly takes place within the framework of the Maillard-reaction, which is a complex multi-step reaction between sugars and proteins. This reaction is favoured in the case of baking and frying but undesired in milk. Here, the lactose reacts with the essential amino acid lysine and therefore leads to a decreased nutritional value. The Maillard reaction already takes place at room temperature but accelerates as the temperature increases. Typical indicators are furosine, 5-hydroxymethylfurfural (HMF), maltol and pyridoxine. The reaction also leads to the formation of brown pigments and a caramel taste caused by maltol, which is especially noticeable for UHT and sterilized milk. The effects are less in pasteurized milk.

Furthermore, the lactose also underlies isomerization: lactulose is formed at higher temperatures, see figure 2.7.

2.2.2 Skimming

Skimming means to separate the fat from the milk. Today, this process is often performed by default when the processed milk is intended for drinking in order to standardize the fat content by adding defined amounts of cream to the skimmed milk later

on. In the following, the mode of operation and the effects on milk are discussed.

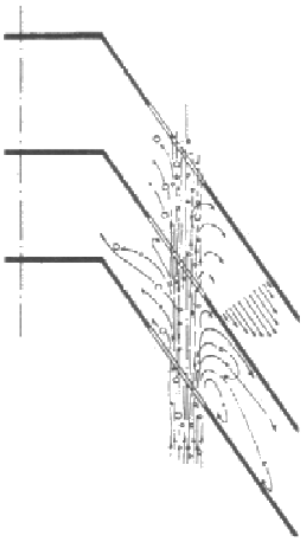


Figure 2.9: Schematic cross-section through a part of a separator disk stack (Tscheuschner, 2004, Abb. 8.10-2a, p. 520).

Mode of operation As mentioned above in section 2.1.1, the density of the fat globules is lower than that of the serum and the caseins. Thus the phases can be separated using high centrifugal forces. Next to skimmed milk and cream, a sediment phase is formed, containing marginal amounts of fat, protein, lactose and dirt particles that are heavier than the milk serum (Töpel, 2004).

The radial force F_R present during separation can be calculated via the following formula:

$$F_R = \frac{m \cdot \omega^2}{R}$$

with m representing the mass of the particle, ω the angular velocity and R the particle's distance from the rotational center (radius).

In the milk the radial forces lead to a separation of particles with a weight difference. Heavier particles are hereby separated faster than lighter particles.

The speed of separation approximately follows Stoke's law. However, instead of the gravitational acceleration g, the centrifugal acceleration $a_z = R \cdot \omega^2$ influence the particles and so the separation speed ν can be calculated as follows:

$$\nu = R \cdot \omega^2 \cdot \frac{(\rho_{pl} - \rho_{fg}) \cdot d^2}{18\eta}$$

whereby $\rho_{pl} - \rho_{fg}$ represents the density difference between the milk plasma and the fat globules and is the crucial factor for the separation, d represents the diameter of the disperse particles (here: fat globules) and η the viscosity of the fluid dispersion

medium (milk serum).

Before skimming, the milk is usually preheated to 40-50 °C to reduce the viscosity and to increase the density difference between the fat and the skim milk. For industrial purposes, the milk is centrifuged in a separator with disc insert, compare figure 2.9. Hereby the milk is divided into thin films, which enhances the separation process. The heavier skim milk phase migrates down during the separating process whereas the lighter cream phase migrates up the disks. Both phases are transported further separately. Usual milk separators work with centrifugal accelerations of about 4-5000 g. Depending on the application, also higher or lower centrifugal accelerations are in use. With higher accelerations, even bacteria and bacterial spores can be removed (Töpel, 2004).

With modern separators the residual fat content of the skimmed milk is smaller than 0.02 % (Tscheuschner, 2004). Only small fat globules with a size < 0.7 μm remain in the skimmed milk. Among others, the obtained fat (cream) is used to produce dairy products such as butter. Usually, a defined amount of cream is mixed with the skimmed milk in order to obtain milk mith a defined fat content. Today, milk is sold as full fat milk ($\geq$ 3.5 % fat), semi-skimmed milk (1.5-1.8 % fat) and skimmed milk ($\leq$ 0.5 % fat).

Effects of skimming on milk Next to the separation of fat from the disperse system milk, some unfavoured side effects can occur during the separation process. If sher stress is high, fat globules can fragment, lower the average size of the fat globules and increase the remaining fat content. Additionally, triacylglycerols can leave the fat globules and are then present as free fat. Free fat is more prone to lipolysis and and the resulting increase of free fatty acids in the milk can cause an off-taste perceived as 'old'.

2.2.3 High pressure treatment

Originally applied in the production of ceramics or other materials like alloys in the beginning of the last century, the usage of HP for food preservation was only given more attention over fifty years later. Today, this non-thermal process is used as an alternative treatment to heating. This means that the food is not heated or cooked while it is processed. Non-thermal treatments usually have one major advantage over thermal treatments: heating leads to nutrient loss and to formation of unfavoured compounds and thus negatively affects food quality, whereas with non-thermal treatments, harmful or food-spoiling micro-organisms are inactivated without or with considerably less adverse impacts (Barbosa-Cánovas et al., 1998).

Basically, HP processing means high hydrostatic pressure is applied evenly throughout a product. Therefore another significant advantage of HP over heat treatment is

that no additional time is needed to obtain evenly distributed pressure like it is the case with heating and temperature distribution throughout the foodstuff.

Fields of application and modes of operation Applied pressures range between 100 - 800 MPa, roughly 1000 - 8000 bar with holding times from seconds to hours depending on the foodstuff and the desired outcome. According to Barbosa-Cánovas et al. (1998) high hydrostatic pressure can be generated in three ways:

1. via *direct compression*, meaning the small-diameter end of a piston pressurizes the medium inside a vessel. Although compression with this method can be very fast, it is mostly used for laboratory applications or pilot plants as there are limitations to the dynamic seal located between the piston and the internal surface of the vessel.

2. via *indirect compression*. Hereby the foodstuffs are shrink-wrapped with flexible foil and put into the vessel that is filled with a pressure medium (Töpel, 2004). The pressure in the closed vessel is increased by an intensifier, which pumps pressure medium into the vessel until the favoured pressure is reached, see figure 2.10. The method of indirect compression is the one most widely used for industry applications.

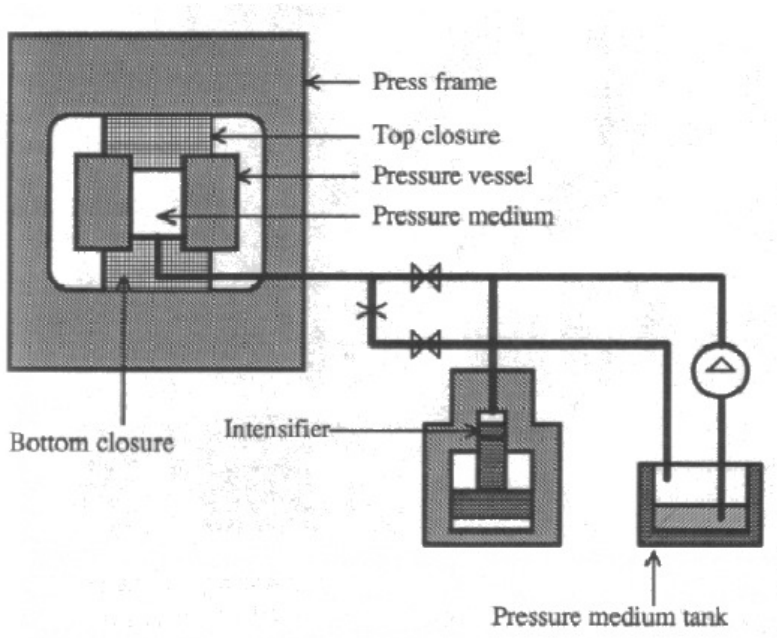


Figure 2.10: Schematic set-up of a HP-unit with indirect compression of the pressure medium (Barbosa-Cánovas et al., 1998, figure 2, p. 11).

3. via *heating of the pressure medium*. With increasing temperature the pressure medium expands and the pressure in the closed vessel rises. This method is used when HP and heat are applied in combination. Very precise temperature control is required throughout the inner volume of the pressure vessel.

Today, high hydrostatic pressure processing is still mostly applied in research labs rather than the food industry. As one of the main reasons one can point out the critical consumers' view on novel food processing techniques (Nielsen et al., 2009). As HP inactivates micro-organisms, enzymes and other proteins in food with only marginal nutrient losses, the potential fields of application in the food industry are huge, ranging from the prolongation of storage time for liquid foods (milk, juices) over prevention of microbial contamination (e.g. fish, meat, eggs or milk) to the development of new food stuffs.

Effects of HP on milk Although HP treatment does not lead to significant nutrient losses, a some structural changes occur during processing. In the following, the main effects on milk are discussed. For more information about the effect on other foodstuffs see for example Barbosa-Cánovas et al. (1998).

Effects of HP on micro-organisms Microbial inactivation is one of the main goals when HP treatment of milk is applied. Firstly, the inactivation of micro-organisms in milk is largely dependent on the processing conditions such as pressure, time, temperature, as well as on the micro-organisms' state (Smelt, 1998). HP, long holding times and high temperature increase the inactivation. Bacteria are more sensitive and therefore more easily inactivated by HP in their exponential growing phase. Bacterial spores are usually more resistant than vegetative cells and can even be stimulated to germinate at pressures ranging between 50-300 MPa. One can use this to inactivate the germinated spores afterwards by mild pressure or heat. Gram-positive bacteria can stand higher pressures and are usually inactivated by application of 500-600 MPa during 10 min at room temperature. Gram-negative bacteria can already be inactivated at 300-400 MPa during 10 min at room temperature (Trujillo et al., 2002). For more details see for example Mussa and Ramaswamy (1997); Barbosa-Cánovas et al. (1998); Smelt (1998); Trujillo et al. (2002).

Effects of HP on proteins After the principle of *Le Chatelier* and *Braun* also called 'The Equilibrium Law', a system in equilibrium experiencing a shift in pressure will always try to counteract the forced change by reactions and other system changes until a new equilibrium is established. This means under HP conditions, reactions are supported that lead to volume reduction. Volume reductions can can be achieved by reactions themselves or by changes of the interactions with water. Pressure stabilizes covalent bonds and hydrogen bonds but weakens hydrophobic and electrostatic bonds. This means that HP only affects the secondary up to the quaternary structure of proteins but not the primary one. (Hinrichs, 2000; Heinrich and Kulozik, 2009)

All proteins hold water free cavities, so called void volumes in the centers of their structures. Furthermore, the side chains of the polypeptide strains are differently

electrically charged. These two properties are responsible for the tertiary structure. At around 200 MPa, the side chains are ionized, ionic and hydrophobic interactions are broken up. The native protein structure is opened and water can fill the former void volumes. With increasing pressure the water can migrate deeper into the protein. The former hydrophobic kernel increases in volume due to the penetrated water. At pressures between 500-1000 MPa the tertiary structure of the protein breaks down and the so called 'molten globule state' is reached where the proteins exist in a orderless state and the water has fully penetrated the protein, which does not contain any void volumes any more. (Gross and Jaenicke, 1994)

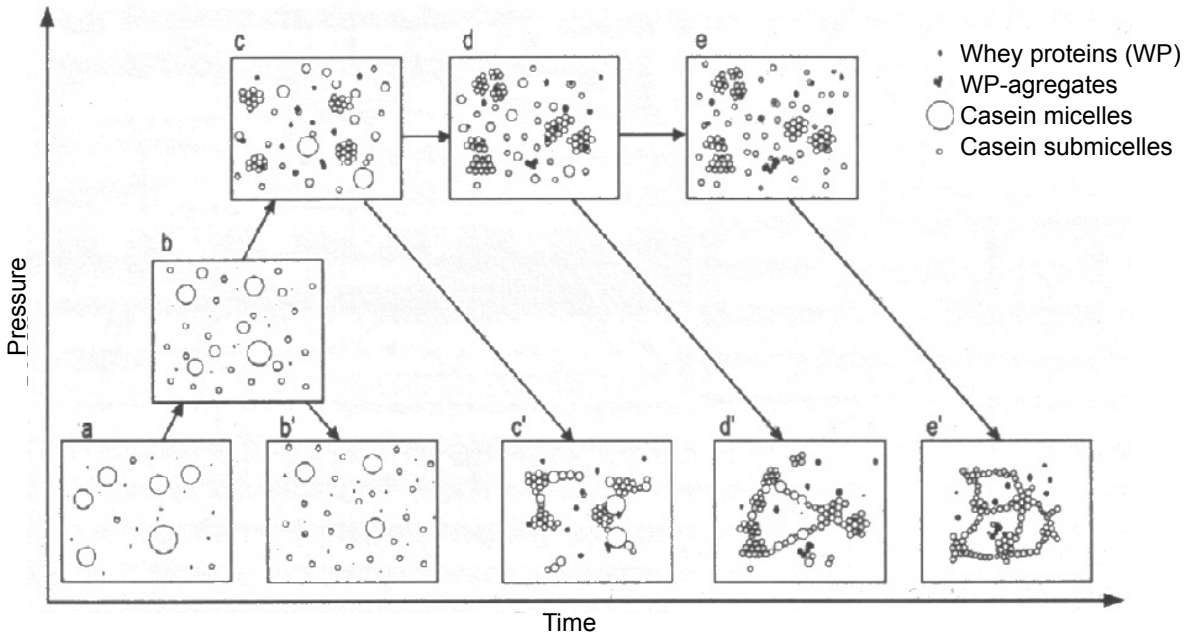


Figure 2.11: Model for the dissociation and aggregation of casein micelles with interactions of whey proteins during HP treatment of milk with pressure generation, holding time and decompression (Hinrichs, 2000, Bild 6.24, p. 134).

Applied to caseins and whey proteins in milk, the following findings were reported: Light transmission of milk increases strongly at pressures ranging from 100-400 MPa, which shows the disruption of caseins visibly for the human eye. One presumes that the calcium phosphate entering into the whey causes the destruction of the casein micelles. from about 400 MPa onwards no calcium phosphate can be detected in the micelles (Huppertz and de Kruif, 2007b,a). This process leads to a disruption of the calcium phosphate clusters and the loss of stability in the casein micelles, compare figure 2.11 a $\rightarrow$ b $\rightarrow$ c (Huppertz et al., 2006). Furthermore, the native micellar structure is disrupted by the loosening of hydrophobic and van der Waals forces (Considine et al., 2007). At pressures lower than 300 MPa the caseins are able to partly re-aggregate again and the turbidity of the milk re-increases, suggesting that not all casein clusters are completely destroyed below this pressure, see figure 2.11 b $\rightarrow$ b' (Huppertz

et al., 2004d). Above 300 MPa however, the casein micelles collapse almost completely and exist as single casein molecules in the serum phase. Re-association happens only partially (c → c'). The resulting particles have the size of about 10-20 nm diameter. Storage temperatures around 5°C prevent a reformation of hydrophobic bonds and therewith the re-aggregation of the casein micelles, which is possible at room temperature (Huppertz et al., 2004a; Huppertz and de Kruif, 2007a).

The denaturation of β-lactoglobulin starts at pressures above 100 MPa. At 400 MPa, around 90 % of the molecules have altered from their native state. Studies have shown that the large part of the β-lactoglobulin molecules binds to casein (micelles). This happens as the quaternary and tertiary structure of the β-lactoglobulin unfolds and the thiol-group exists in a free state. This thiol-group then reacts with κ-casein, but also with α-lactalbumin, another unfolded β-lactoglobulin molecule (compare figure 2.11 c → d → e) or milk fat globules. The formation of new disulfide bonds leads to protein aggregation, which continues to exist after decompression, see figure 2.11 c → c', d → d', e → e'. After decompression, those β-lactoglobulin molecules that did not form a disulfide bond with another molecule are able to fold back into their native state. (Huppertz et al., 2004c,b; Hinrichs and Rademacher, 2005; Huppertz et al., 2006; López-Fandiño, 2006)

α-lactalbumin is more resistant to pressure induced denaturation than the other milk proteins mentioned. Denaturation starts around 400 MPa and only 70 % is denatured at 800 MPa(Huppertz et al., 2004c,b). The increased stability can be explained by the more compact molecular structure, a higher amount of intra-molecular disulfide bonds and the lack of free thiol-groups. (Hinrichs et al., 1996; López-Fandiño et al., 1996; Gaucheron et al., 1997). During denaturation, the hydrophobic kernel of the α-lactalbumin molecule is destroyed (Considine et al., 2007). As mentioned above, α-lactalbumin can form aggregates with β-lactoglobulin under pressure, also when α-lactalbumin still exists in a native state. However, denaturation and aggregation of α-lactalbumin are dependent on free thiol groups as they occur with denatured β-lactoglobulin (Jegouic et al., 1996).

The changes discussed above for proteins also apply for enzymes, which react differently to the induced pressure. For example, Scollard et al. (2000) found that the activity of plasmin, a proteinase, was reduced at 400 MPa. Elevated temperatures increased the effect. Some more effects on enzymes were detected. For more details see Seyderhelm et al. (1996); Huppertz et al. (2002).

Other effects of HP It was observed that HP treatment also affects the milk fat: leading to its crystallization. The strongest effect was observed at 200 MPa. However, morphological properties of the crystals are the same as when formed at atmospheric pressure and even very HP does not lead to destabilization of the fat globules (Huppertz et al., 2002). Already at relatively low pressures (around 100 MPa), denatured β-

lactoglobulin molecules associate with the membrane proteins of the fat globules. This effect increases with pressure and treatment time. Above 500 MPa, the same applies to κ-caseins and α-lactalbumin (Ye et al., 2004). This association leads to retarded creaming of HP treated milk (Huppertz et al., 2003). It was also found that HP might induce light autoxidation of milk fat, especially linolenic acid[3]. However, compared to heat induced autoxidation, no new compounds were found (Butz et al., 1999).

Another effect of HP treatment was already mentioned previously: An increased amount of free ions in the milk serum originating from the calcium phosphate dissociated from the casein micelles (López-Fandiño et al., 1998).

2.2.4 Pulsed electric fields treatment

In the last few decades, the popularity of the PEF treatment has increased steadily, mostly among research groups. PEF treatment is still not used widely in the food industry, mainly out of problems with consumers' acceptance (Nielsen et al., 2009). The PEF treatment is another non-thermal treatment applied to foodstuffs that is able to prolong the shelf life without the unfavourable side effects of the heat treatment. In the following, operational processes and effects of PEF treatment on the system milk are discussed.

Mechanism of action A high intensity electric field applied for only a few microseconds has the ability to induce structural changes and cell membrane breakdown of organic tissue. The PEF treatment is based on this phenomenon, called electroporation. The electroporation can be reversible at lower intensities. In the field of genetics, this is currently used to transfer foreign material such as DNA into plant tissue or micro-organisms. At higher intensities, membrane permeabilization is irreversible and can be used to inactivate micro-organisms (see below and in figure 2.12) or for the disintegration of plant tissue for example to increase the rate of yield in juice production. (Barbosa-Cánovas and Altunakar, 2007)

There are many theories about the mechanism of pore formation in the cell membranes, its reversibility or irreversibility. According to Sale and Hamilton (1968), the development of a trans-membrane potential across the cell membrane during exposition to an external electric field is crucial to the formation of pores. Crowley (1973) developed a theory of electromechanical instability causing pore formation and microbial cell death: naturally, the cell membrane is considered to own a perpendicular transmembrane potential of about 10 mV with accumulated charges of opposite polarity (+ and -) on the two sides of the cell membrane. When an applied external electric field induces an additional potential, charges are moved along the electric field lines. This leads to a viscoelastic deformation of the cell membranes. Above a critical value of

[3] an essential omega-3-fatty acid

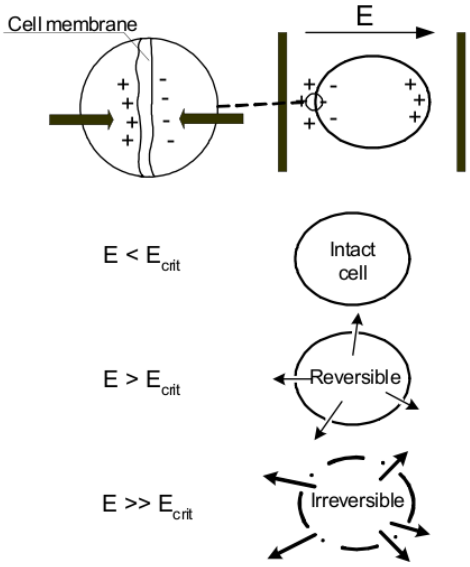


Figure 2.12: Schematic depiction of mechanism of membrane permeabilization by electro-compressive forces induced by an external electrical field (Töpfl, 2006, Figure 2.7, p. 14).

the potential (about 1 V), pore formation is induced by the electro-compressive force, which causes a local dielectric rupture in the cell membrane, see figure 2.12. The pore hereby then acts as a conductive channel (Crowley, 1973; Zimmermann et al., 1974; Schoenbach et al., 1997; Töpfl, 2006). Other theories include molecular reorientation and local defects inside the cell membrane. Exposure to PEF is said to expand and destabilize these defects. For more information see Glaser et al. (1988); Chang et al. (1992).

PEF equipment and mode of operation Basically, the treatment of PEF includes pulses of short duration (1-10 μs) of a high electric field strength applied to a food, which is placed between two electrodes. For the pulse generation the following equipment is usually needed: a high voltage power supply, an energy storage element (capacitive or magnetic), a switch, a treatment chamber, additionally a pump if the treatment is conducted in flow and an oscilloscope for pulse waveform observation. A typical high voltage pulse generator system, which creates exponentially decaying pulses, is shown in figure 2.13.

The power supply creates a high voltage direct current (DC). This energy is stored in one or several capacitors until it is discharged through the treatment chamber using a high-voltage switch, which is able to work at high repetition rate and power. The energy Q, which is stored by the capacitor can be calculated as follows: $Q = \frac{CU^2}{2}$, with C being the capacitance and U the charging voltage. The charging of the capacitor usually takes about 1 to 100 μs. The treatment chamber consists of (at least) two electrodes, usually made out of metal, separated by insulating material. Treatment chambers in various

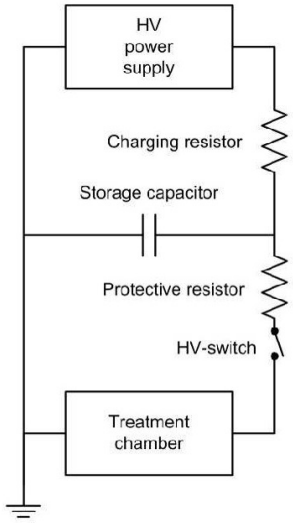


Figure 2.13: Scheme of a high voltage pulse generator which creates exponentially decaying pulses (Töpfl, 2006, Figure 2.9, p. 27).

different shapes and arrangements have been developed. One of the most common arrangements used for laboratory purposes is the parallel plate chamber. The size of the electrode surface S and the inter-electrode gap d_i are two parameters affecting the resistance of the treatment chamber R_a in the following relationship: $R_a = \rho_a \cdot \frac{d_i}{S}$, with ρ_a being the electrical resistivity of the sample. As one can see in figure 2.13, the high voltage switch has two positions, open and closed. At a opened position, current flows into the capacitor and it is charged. Then, the switch closes, the capacitors are discharged and current flows into the treatment chamber and generates an electric field (Töpfl, 2006). The generated pulses can have different shapes, for example a square-like wave-shape, an exponentially decaying shape or bipolar pulses with alternating polarity depending on the type of switch used (Barbosa-Cánovas et al., 1999).

Various different processing parameters exist in PEF processing: the electric field strength E , the specific energy W_s which is related to the energy per pulse W_{pulse} , the treatment time t, frequency f, pulse geometry and pulse width τ.

The electric field strength E is an important parameter for generating cell disruption. It depends on the distance between the electrodes d, its geometry and on the voltage U and can be calculated by the following formula: $E = \frac{U}{d}$. Research has shown that a field strength of about 1-2 kV/cm is necessary to cause cell permeabilization of plant and animal cells. A higher intensity of about 10-15 kV/cm is necessary for electroporation of microbial cells (Töpfl et al., 2007). The specific energy W_s describes the quantity of electric energy that the processed food receives and is specified per kilogram of food. It is a useful tool to compare the energy consumption of a PEF process with more traditional processes. For batch treatments it can be calculated as follows: $W_s = W_{pulse} \cdot \frac{n}{m}$ with n the number of pulses and m the mass of the processed

food. For continuous treatments, $\frac{n}{m}$ must be replaced by $\frac{f}{\dot{m}}$ with f the frequency and $\dot{m}$ the mass flow rate. W_s can also be calculated based on the conductivity of the medium σ and E. For more details see Töpfl (2006). The determination of the energy per pulse W_{pulse} is specific for the pulse shape. For example, for exponentially decaying pulses, it is estimated by the energy that is stored by the capacitor: $W_{pulse} = \frac{1}{2} \cdot C \cdot U^2$ with C being the capacity of the capacitors and U the initial charge voltage. Next to this, treatment time t and frequency f are also important parameters to consider, in order to obtain the favoured total energy input and to avoid under- or over-processing. The treatment time can be calculated as the product of pulse duration τ and the number of pulses n: $f = \tau \cdot n$. As mentioned above, differently shaped pulses can be generated depending on the switch. Qin et al. (1994); Zhang et al. (1994) found that square-wave pulses inactivate micro-organisms most effectively, pulse generating, however, is rather complex and costly. On the other hand, exponentially decaying pulses are more easy to generate, but less effective. For exponentially decaying pulses, the pulse width is defined as the time measured at which E is reduced to 37 % of the initial value.

Effects of PEF on milk The PEF treatment is considered to be a good alternative for heat treatment of milk, as structural changes observed at high temperatures do not occur or are less intense. With the PEF treatment it is possible to pasteurize foods including milk at temperatures below 30-40 °C (Barbosa-Cánovas and Altunakar, 2007). Effects on micro-organisms and milk substances are discussed below.

Inactivation of micro-organisms Many studies have been performed to assess the effectiveness of PEF treatment on the inactivation of various different micro-organisms on a large number of food stuffs, including milk. According to Barbosa-Cánovas and Altunakar (2007) the applied electric field strengths mainly range from 10-80 kV/cm. Other parameters and equipment design differ widely among the studies, which makes a comparison rather difficult. Some results are mentioned in the following. For example, Qin et al. (1995) reported that microbial shelf life of milk could be prolonged up to about two weeks after PEF treatment, investigating different treatment conditions. Bendicho et al. (2002) reported an inactivation of *Staphylococcus aureus* of about 4 log cycles. Coagulase negative *Staphylococcus sp.* was found to be inactivated 2 log cycles. However, they did not observe any significant reduction of *Corynebacterium sp.*, *Xanthomonas maltophilia* and others. However, processing parameters were not specified. Jäger et al. (2009b) reported a 2 log inactivation of *Lb. rhamnosus* with a field width of 30 kV/cm at an energy input of 116 kV/cm in raw whole milk. Eberhard and Sieber (2006) gathered a detailed overview of various more results of microbial inactivation due to PEF treatment.

Effects on substances in milk Barbosa-Cánovas et al. (1998) found that no changes in the chemical of physical properties of milk occurred after PEF treatment. It remain unclear, however, what aspects they refer to.

As Bendicho et al. (2002) reported, the inactivation of enzymes in milk requires higher energy input than the inactivation of micro-organisms. Inactivation depends on the intensity of the electric field, the number of pulses, enzyme characteristics and the concentration of the enzyme. For example, Grahl and Märkl (1996) observed the strongest effects on lipase (activity loss of 50 %), a slight effect on peroxidase (-30 %) and almost no effect on alkaline phosphatase (-7.5 %) at at electric field strength E of 21.5 kV/cm with 20 pulses applied.

Whereas various studies have been performed on the effect of PEF treatment on micro-organisms, the number of conducted studies on the influence on substances in milk is still rather small. In the case of vitamins, no significant losses were found except for vitamin C. Grahl and Märkl (1996) reported losses of up to 90 % of the original content. The effects of PEF on milk proteins remain unclear: whereas Michalac et al. (2003) found no changes in the protein constitution, Floury et al. (2005) observed an effect on caseins: With a maximum field strength between 45-55 kV/cm and temperatures below 30 °C the size of the casein micelles decreased, suggesting the effect to be "probably due to modification of the apparent charge after exposure to intense electrical fields and then modification of the ionic interactions between the caseins" (p. 55). Barsotti et al. (2001) studied the effect of PEF on β-lactoglobulin. They did not discover any signs of aggregation or unfolding. Also, the size distribution of fat globules did not alter. However, Perez and Pilosof (2004) found a decrease of up to 40 % of β-lactoglobulin in a watery solution at an electric field of 12.5 kV/cm with ten pulses and a treatment time of 15 s. One can hypothesize that other substances present in milk protect the β-lactoglobulin from denaturation. Odriozola-Serrano et al. (2006) did not detect significant differences for β-lactoglobulin, α-lactalbumin or bovine serum albumin in whole milk treated at a field strength of 35.5 kV/cm applied up to 1,000 μs.

Many authors, for example Morren et al. (2003); Roodenburg et al. (2005); Saulis et al. (2007); Jäger et al. (2009a) reported the occurrence of other factors such as temperature increase, electrochemical reactions or local pH shifts that occur during PEF treatment and which might have a negative influence on food and in this case, milk compounds. Especially the impact on sensitive compounds such as enzymes and other proteins might be high as they are sensitive to local pH changes (Jäger, 2011).

2.3 High-performance liquid chromatography

HPLC is a modern standard analytical method used to detect substances in a sample with high accuracy and sensitivity. Below, principles of chromatography and the general HPLC-setup are presented.

2.3.1 Principles of chromatography

Chromatography is a process whereby the compounds in a sample mixture are separated due to differences in their portioning behavior between a mobile phase and a stationary phase within a separating segment. The mobile phase flows through the stationary phase and carries the sample mixture with it. The stationary phase is either solid or liquid. Solid materials are for example agarose, porous aluminium oxide or silica gel. The mobile phase either a liquid, which is not solvable in the stationary phase, or a non-soluble gas. The mobile phase can also be a mixture of several liquids. Its composition during the experiment can stay the same (isocratic elution) or change (gradient elution). (Matissek and Steiner, 2006; Meyer, 2009)

Nowadays, there exist many types of chromatography, which can be divided according to different principles: the mechanical construction of the separating segment, the combination of the different phase types or the type of distribution (Matissek and Steiner, 2006).

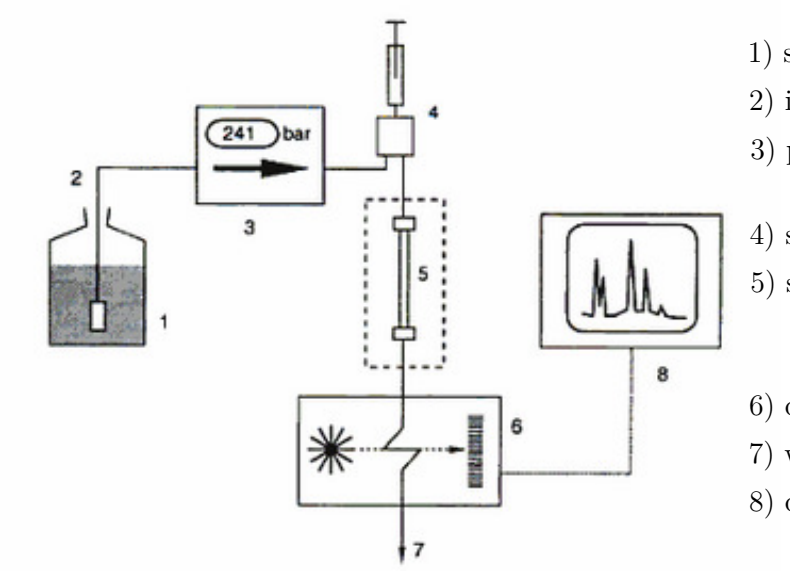
In the case of liquid-solid-chromatography, where the stationary phase is solid and the mobile phase liquid, several different types can be distinguished, for example the ion-exchange chromatography, affinity chromatography, size exclusion chromatography or liquid adsorption chromatography (Meyer, 2009).

There exist now types of liquid adsorption chromatography: normal phase chromatography (NPC) and reversed phase chromatography (RPC). Generally speaking, the separation results from the difference in polarity of the compounds in the sample mixture and the different phases (Heisz, 1987). For the NPC, the stationary phase consists of a polar material, such as silica gel with an apolar mobile phase. As the name indicates, the reverse applies to RPC. Here, a polar mobile phase is used and the stationary phase is made apolar by modification with chemically bound alkyl groups of different lengths. The amount of C atoms is mentioned as a characteristic attribute, for example C18, means, the alkyl rest contains 18 C-atoms. (Meyer, 2009).

High-liquid-performance-chromatography is a type of liquid-solid-chromatography whereby the separating segment consists of a thin column filled with particles of small diameter, around 3-30 μm. During the experiment the pressure rises up to about 300 bar, which gives this type of chromatography its name.

2.3.2 HPLC setup

Figure 2.14 shows a typical setup for an HPLC-system with isocratic functioning. It consists of the following components: a solvent supply, inlet pipe and frit, a pump, the sample injection, separating column, detector, and waste container with inlet and a computer for data collection. Additionally, a degasser and a gradient forming system are necessary for gradient elution.



1) solvent supply
2) inlet pipe with frit
3) pump (with digitally displayed pressure)
4) sample injection
5) separation column (potentially thermo-statted)
6) detector
7) waste
8) data collection

Figure 2.14: Scheme of an HPLC equipment with isocratic functioning (Meyer, 2009, Abb. 1.3, p. 11).

Solvent supply The solvent supply contains the liquids for the mobile phase. One ore several eluents can be mixed for chromatography. Usually, two different eluents, for example water and acetonitrile are used. Via an inlet pipe, the solvents are delivered further by means of a pump (see below). The inlet pipe contains a frit with holes of usually 0.2 μm to prevent very small particles from entering the system. (Meyer, 2009)

Degasser The degasser is used for gradient elution and degases the eluents online before mixing by carrying them through membrane pipes, which are gas permeable by applied vacuum. The gas flows through the membrane into the vacuum. Degassers are usually added in front of the pump. (Meyer, 2009)

Pump Today, the most commonly used pumps for HPLC are double piston pumps. Principally, the mobile phase is pushed through a one way valve with the aid of a piston. Two pump heads are connected offset so that one pump head carries the eluent and the other simultaneously draws in eluents from the solvent supply. This guarantees a pulsation free flow. Double piston pumps are especially suitable for the gradient elution. Another pump type is the displacement pump, which transports the solvent phase by pushing the piston slowly forward. These pumps manage to work with high flow rates up to about 10 ml/min. Another advantage is that they work without valves and therefore with a pulsation free flow, which is however only reached after a long starting period. This pump type is not suitable for gradient elution. (Schwedt and Vogt, 2010)

Gradient forming system The gradient forming unit is crucial for the gradient elution as it mixes the different eluents according to preselected time dependent concentrations.

Two types of gradient forming systems exist: low and high pressure gradient systems. In the case of the low-pressure gradient system, the eluents are transported into a mixing chamber and carried further via a pump into the separation column. In contrast, the high-pressure gradient system mixes the eluents directly before they enter the column. (Meyer, 2009)

Sample injection Nowadays, the samples are usually injected by an auto-sampler, which injects the samples automatically according to a programmed sequence. It is also possible to inject the samples by hand with an injection valve. (Meyer, 2009)

Separating column The separating column is the core of every HPLC system and selection of the right column is crucial to obtain meaningful results. Generally, it is important to select a column which shows great chemical, thermal and mechanical stability. Other important characteristics are an equal particle size and a high permeability for the mobile phase. Usually, columns consist of stainless steel, however also other HP resistant materials are in use. Inner diameters and length of the columns range between 3-10 mm and 10-25 cm, respectively. A precolumn can be used to prolong the durability of the column. It protects the column from contamination and the dissolving of silicic acid from the silica gel via the mobile phase. (Heisz, 1987; Meyer, 2009; Schwedt and Vogt, 2010)

Detector and data collection Basically, the detector is the part of an HPLC-system where the single substances of the sample mixture are determined. It detects the change in composition of the mobile phase and processes the signals further to a computer where data is collected and displayed in chromatograms (see section 2.3.3). For good detectors, the signal interference should be as small as possible and detection should be very sensitive and robust at the same time. There exist different types of detectors, which are classified according to the properties they determine.

The most commonly used detector is the UV/Vis-detector, which determines the change of the irradiated light at a certain wavelength when passing the sample. Different lamp types produce light of different wavelengths. For example, deuterium lamps emit light with a wavelength-spectrum up to 340 nm, whereas the emitted light spectrum of tungsten lamps lies between 340-850 nm. So called diode-array-detectors (DAD) are special UV/Vis-detectors that are able to record a wide band of the spectrum simultaneously. (Meyer, 2009; Schwedt and Vogt, 2010)

2.3.3 The chromatogram obtained by HPLC

For the evaluation of the data obtained by the HPLC experiments, so called chromatograms are used. An example of the basic principle of a chromatogram is shown

in figure 2.16. The different components are shown in different peaks. On the basis of these peaks, the detected substances can be identified and quantified. Hereby, the area under each peak and its height represents the relative concentration of the compound, see figure 2.15. With the aid of a standard curve for purified substances that was obtained beforehand, one can calculate the actual concentration of the compound in the sample.

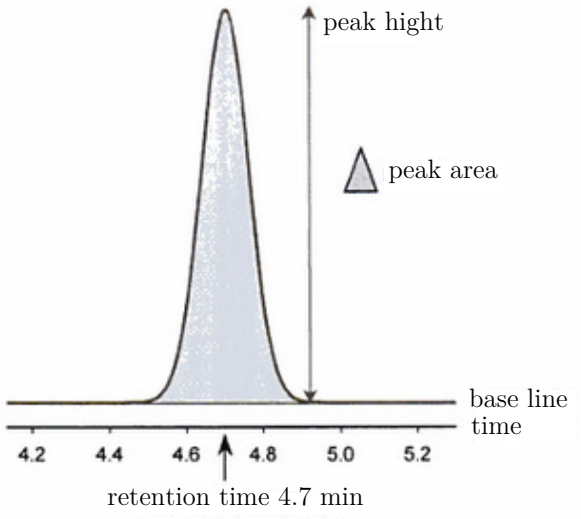


Figure 2.15: Characters of a peak in an HPLC-chromatogram (Meyer, 2009, Abb. 2.8, p. 24).

One can identify the substances by means of their retention time, compare figure 2.16. The retention time is specific for each substance and method used, therefore it is only possible to identify substances with standards when the same methodology is applied. The retention time, also called gross retention time t_{Ri} is the time elapsed between the sample injection and the peak maximum of substance i. The net retention time t'_{Ri} can be calculated with the aid of t_{Ri} and t_0, the dead time via the following formula: $t'_{Ri} = t_{Ri} - t_0$. Hereby, the dead time is the time the mobile phase needs to flow through the separation column. Thus, net retention time is the time the substance i dwells inside the separation column. It is specific for every substance.

2.4 Picture forming methods

PFMs were first thought of by Rudolf Steiner, the founder of biodynamic agriculture and anthroposophy[4] in the beginning of the last century as alternative methods for the detection of food quality. Since then, the methods have been developed further, for example by Pfeiffer (1930); Selawry and Selawry (1957); Engquist (1970); Knorr and Vogtmann (1983); Beckmann et al. (1993); Zalecka (2006); Kahl (2007).

[4]Antroposophy comes from the Greek words ánthropos 'human' and sophía 'wisdom'.

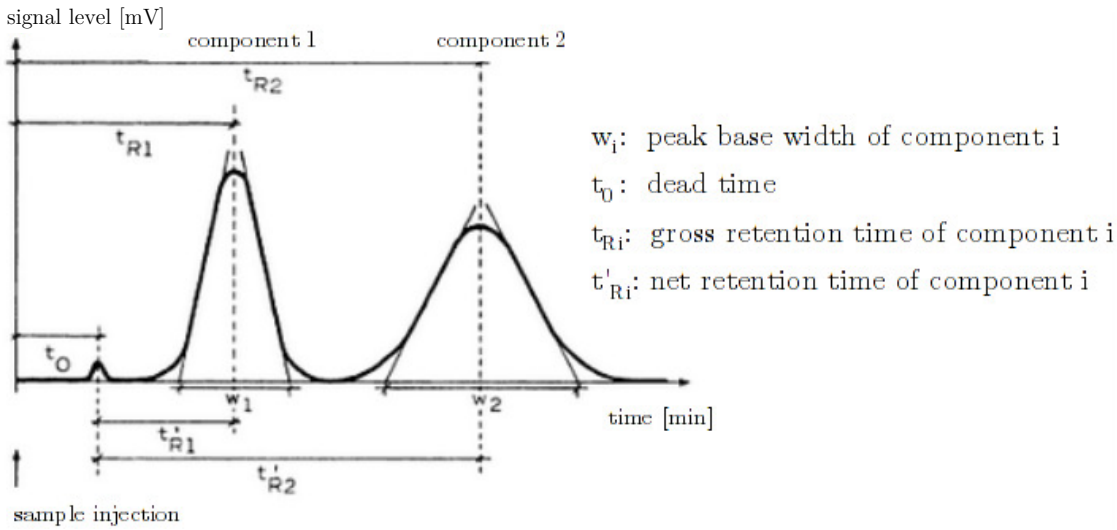


Figure 2.16: The HPLC-chromatogram and its parameters (Meyer, 2009, Abb. 2.9, p. 25).

Modern general science and the alternative, so called 'holistic' science differ in their general approach. Whereas general science is based on induction, namely to obtain greater conclusions from many partial tests and outcomes, holistic science derives its conclusions via deduction, which is the inference of partial truths from general state of affairs. One can call the the first a 'bottom-up'- and the latter a 'top-down'-approach, compare figure 2.17. Hence, today's general science is good at analysis, which involves the breaking up into aspects and parts but is bad at synthesis. Usually, modern science uses statistics to partly bridge the gap.

In contract to today's general science, supporters of the PFM and holistic science follow a top-down approach and claim that "the whole is more than the sum of its parts" (Aristotle), meaning that the properties of food cannot solely be described by the sum its components but that there is 'something that holds these parts together' which cannot be reduced to its single parts (as with the bottom-up approach). A carrot, for example, consists of atoms, molecules and cells which interact among each other. However these components alone do not form the carrot: there exists a fundamental leap between the carrot as a whole and its single parts, compare figure 2.17). It is this 'something that holds the parts together' that the researchers aim to detect with the PFMs and is referred to by some authors with terms like 'vital quality' (Balzer-Graf, 2001; IFOAM, 2009), 'inner quality' (Bloksma et al., 2003), 'order' (Schrödinger, 1944) or 'structure' (Kusche et al., 2010). This order might be altered during aging or processing and thus 'disorder' can be created (see also Abel (2012)).

The supporters of PFMs claim to illustrate this 'order' and 'disorder' in the created images with the aid of the *whole* food sample (top-down), instead of splitting the sample up into ever smaller parts (bottom-up). The only sample preparation used is simple extraction, for example the pressing of juice from an apple. For this reason,

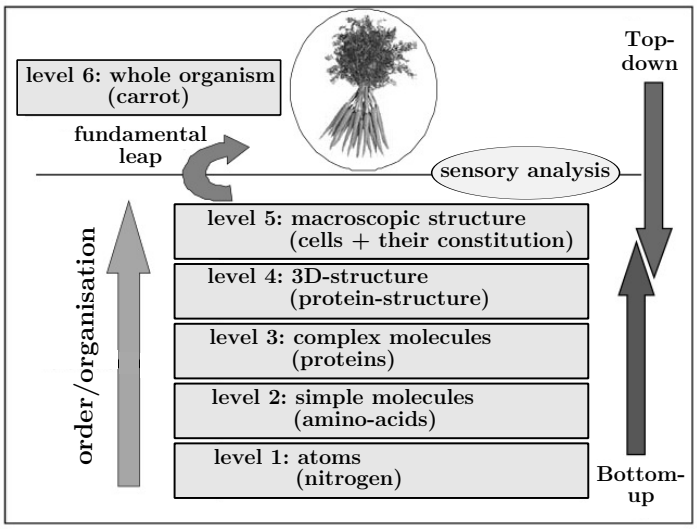


Figure 2.17: Model of structure-levels for the example of a carrot (Kusche et al., 2010, figure 1, p. 100).

PFMs are called 'holistic'. In contrast to standard analytical methods, methodology is rather simple. Most PFMs are based on chromatography or crystallization and only salt solutions are used additionally.

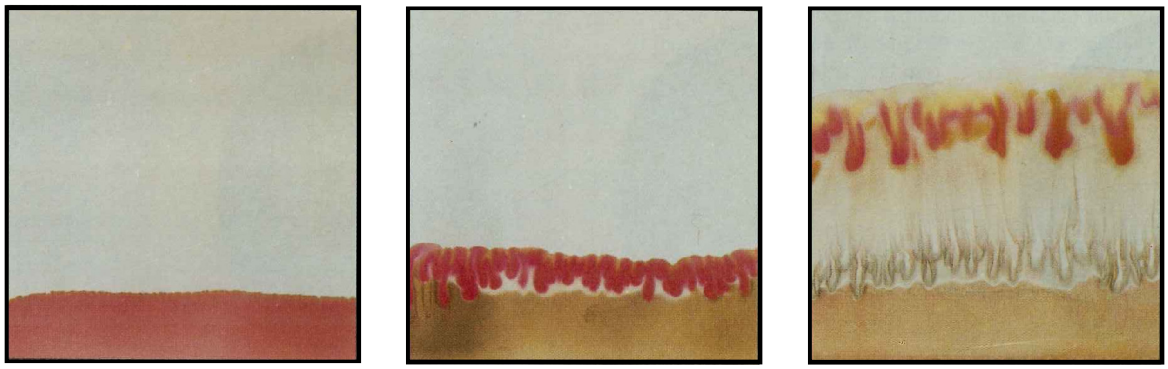
An issue remains the interpretation of the pictures obtained. Traditionally, the pictures were and still are evaluated visually with self-chosen criteria and judgments. As the concept of 'structure' or 'order' of the food is not graspable with conventional methods and approaches and the visual evaluation has been rather subjective, PFMs have not been scientifically accepted by general science for a long time. Only in the last two decades, research groups in Denmark, Germany and the Netherlands started to validate the methods for certain purposes, for example the differentiation between crops grown with different farming methods. Hereby they also developed a more objective visual evaluation, based on sensory norms and a computerized texture and structure analysis (Andersen et al., 1999; Zalecka, 2006; Kahl, 2007).

The potential of PFMs lies in the additional value added to the outcomes of the standard analytical methods of today's general science. Ideally, when both types of research are combined, the sum of parts detected by general science plus the extra information gained with PFMs, will fully be able to describe the whole.

In the following, the PFMs Steigbild, circular chromatography and biocrystallization are described in more detail. Next to these, there are many other holistic methods for food quality detection such as biophoton analysis (Popp, 1991; Popp and Belousov, 2003; Strube and Stolz, 2004), electrochemical analysis or storage tests. For more details see Meier-Ploeger and Vogtmann (1991).

2.4.1 The Steigbild method

The Steigbild method (also called rising picture method or capillary dynamolysis) was already developed in 1923 by Lili Kolisko and Rudolph Hauschka[5] upon the advice and inspired by the ideas of Rudolf Steiner, to study "Gestaltungskräfte" (English: creative powers) (Zalecka, 2006, p.9). It is mainly used for quality tests of agricultural produce, mostly of plant origin and for quality tests of herbal medicines. It has been validated for the differentiation of farming methods and processing steps for several crops (Zalecka, 2006). In the past, the Steigbild method has also been applied for the early diagnosis of cancer (Kaelin blood test) and to study the effect of planetary constellations on plants. (Fyfe, 1967, 1973; Steffen, 1983)



(a) (b) (c)

Figure 2.18: The three phases of the Steigbild method: (a) the first phase: beetroot juice rises up to about 3 cm, (b) the second phase: $AgNO_3$ rises up to 1 cm higher and (c) the third phase where $FeSO_4$ rises up to about 12 cm and forms the final picture after exposure to diffuse light (adapted from Balzer-Graf and Balzer, 1991, figure 2, p.193).

Methodology Basically, the Steigbild method is a chromatographic method whereby food extracts and salt solutions subsequently rise up a filter paper. Thereby, certain characteristic shapes are formed. For a long time, methodology has not been standardized. This only changed with the PhD-thesis of Zalecka (2006). The description of the Steigbild procedures in this thesis follow her work.

The sequence of the Steigbild method contains four steps: Firstly, the preparation of a watery (plant) extract via chopping and pressing or when the sample is a liquid, usually dilution with distillated water. The three rising phases subsequently follow:

A small amount of sample extract or dilution rises up to around 3 cm on a special filter paper standing rolled up in a special dish (Kaelin-dish), compare figure 2.18 (a). The rising happens due to capillary forces under controlled temperature and

[5]the founder of the Wala Heilmittel GmbH, a company that in specialized in anthroposophic medicine and cosmetics, for example under the brand name 'Dr. Hauschka' (see WALA Heilmittel GmbH, 2012)

atmosphere and in darkness as early picture developing due to light has to be avoided. compare . After drying, a solution of silver nitrate ($AgNO_3$) rises approximately 1 cm higher than the sample and let dry again (figure 2.18 (b)). During the rising of the $AgNO_3$ solution, a high beaker is put over the filter and the Kaelin dish to increase humidity, which leads to faster rising. In the third rising phase a ferrous sulfate solution ($FeSO_4$) is used, rising up to about 12 cm on the filter paper, again covered with a beaker. After another drying period, the filters are unrolled and are exposed to diffuse light conditions for colour development. During development, the colours become darker. A developed example of a Steigbild chromatogram is shown in figure 2.18 (c). For more details see Balzer-Graf and Balzer (1991); Zalecka (2006); Zalecka et al. (2010).

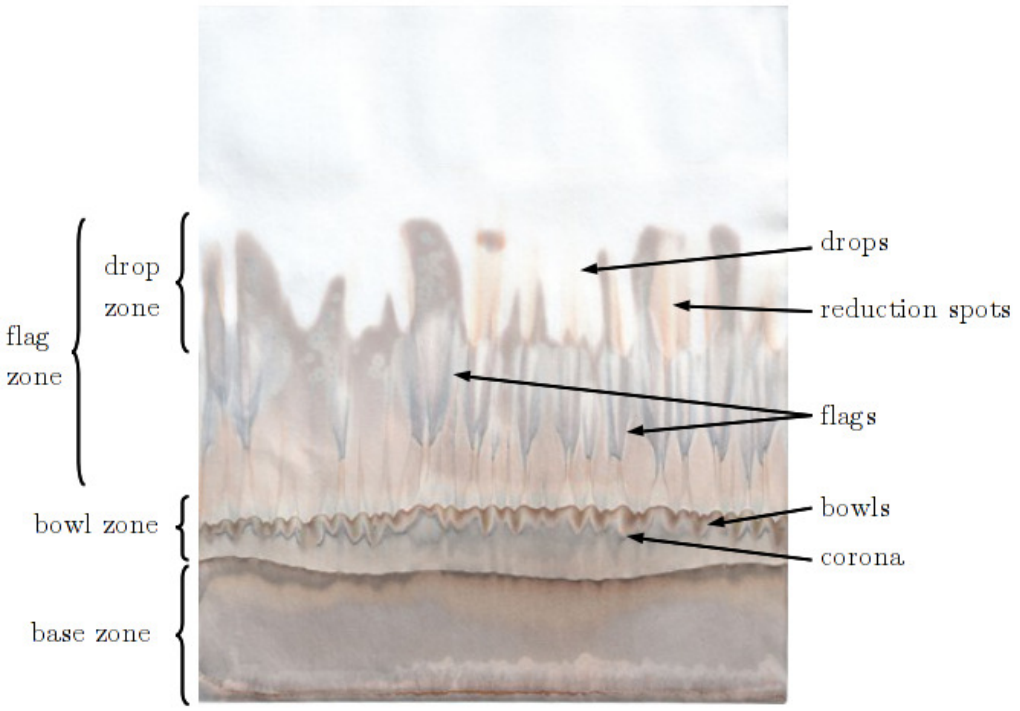


Figure 2.19: Description of zones of a Steigbild with some criteria for visual evaluation on the basis of Zalecka et al. (2010, figure 1, p. 47).

Picture evaluation Strongly subjective in the past, Zalecka (2006) developed an objective visual analysis of the pictures which follows DIN- and ISO-norms for sensory analysis, namely the 'simple descriptive test' (DIN 10964:1996) and the 'triangular test' (DIN EN ISO 4120:2007) which were adapted to the Steigbild method. The picture is interpreted after morphological criteria for each of the three zones base zone, bowl zone and flag zone that were classified according to the difference in pattern, compare figure 2.19. To check the reproducibility, several Steigbild images per sample are produced and evaluated simultaneously according to the 'simple descriptive test' via a trained panel also comparing them to pictures obtained before from other experiments.

With the 'triangular test' one can test whether and how significantly two samples of different origin can be distinguished from each other. Hereby, three pictures are shown simultaneously in a triad, from which two originate from the same sample (double picture). The task is to recognize the odd one out. Statistical analysis can be carried out via a statistical program and with the use of the tables in the appendix of DIN EN ISO 4120:2007. (Zalecka, 2006; Zalecka et al., 2010)

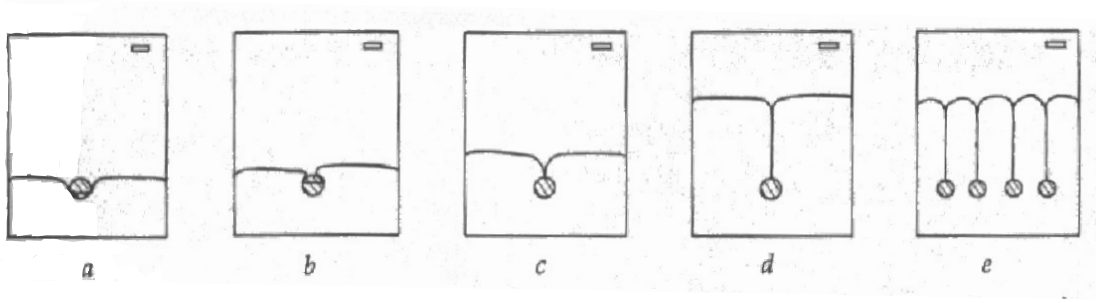


Figure 2.20: Induction of a vertical route of reduced silver through local flow delay of the $AgNO_3$ solution due to dried albumin, explanation see text (Steffen, 1983, Bild 6, p. 45).

Physical-chemical principles of pattern formation Steffen (1983) tried to explain the development of specific forms like bowls, drops and flags, compare figure 2.19. The differentiation of colours and forms evolves due to different interactions of the dried substances of the sample (extract) from the first rising phase and the rising metal salt solutions with subsequent exposure to light.

Figure 2.20 a to d shows the rising behaviour of $AgNO_3$ when confronted with a drop of dried albumin. In figure 2.20 one can see the the effects of several albumin drops aligned in a horizontal row. The results show that the dried albumin drop acts as a local flow delay, which leads to the formation of a boundary line between two adjacent zones of flow. The boundary line is marked with reduced silver atoms which are then permanently adsorbed and show a brownish to greyish layer in the form of bowls (see figure 2.19). When the $AgNO_3$ solution crosses the flow limit of the dried substance or sample, the flow-pattern above this boundary becomes differentiated and leads to the formation of the flags, compare figure 2.19.

Solubility- and desorption-properties of the dried substance(s) are involved in another process leading to the development of Steigbild specific forms: figure 2.21 shows the formation of droplets using the example of a dried sugar solution. When the $AgNO_3$ solution reaches the dried sugar, it solves the sugar at the front. It is then lodged in vertical lines called tracks. Due to the increased viscosity, the further rising of these tracks is delayed, see figure 2.21 a. The continuous subsequent flow of $AgNO_3$ forms those tracks into drawn-out drops, see figure 2.21 b. During the rising of the $AgNO_3$ two phases occur: a silver free front and a silver containing fraction.

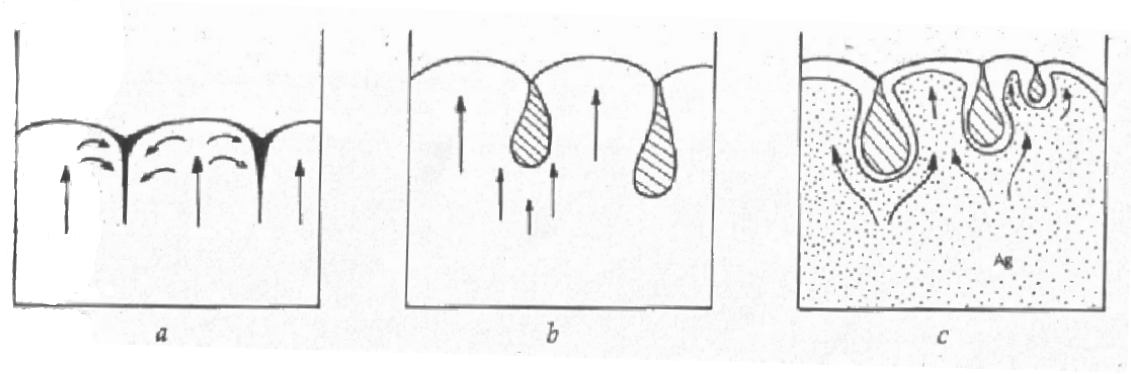


Figure 2.21: Differentiation and development of forms on the rising $AgNO_3$ front, explanation see text (Steffen, 1983, Bild 7, p. 45).

Solubilization of the substance (here sugar) happens almost solely in contact with the silver free fraction. The silver free fraction mostly 'avoids' these fractions and rises up the substance free parts of the paper, see figure 2.21 c. For other substances than sugar, the occurrence of desorption and distribution equilibria of the substances lead to flow delay. When $FeSO_4$ is added, the following reaction takes place:

$$2AgNO_3(aq) + FeSO_4(aq) \longrightarrow Fe(NO_3)_2 + Ag_2SO_4(s)$$

The formed silver sulfate ($AgSO_4$) darkens under exposure to light or air, which explains the occurrence of brownish colours, compare figure 2.19. Furthermore, $AgNO_3$ is reduced to darkish silver (Ag) when it reacts with organic compounds or dust, especially in the presence of light. It can be guessed that a variety of different reactions take place that are not yet discovered, which lead to the formation of the different colours observed in the single zones next to the occurrence of differently shaped patterns (Steffen, 1983). During processing of food modification of substances and / or formation of new compounds occur. (compare section 2.2). These changes should be visible or detectable in the picture.

Effects of milk treatments on pattern formation Until now, most research that was conducted using the Steigbild method has been done on plants (for example Balzer-Graf and Balzer, 1991; Zalecka, 2006; Zalecka et al., 2010). Only a few authors dealt with the influence of different factors on the pattern (and colour) formation of chromatograms obtained from milk.

(Balzer-Graf and Gallmann, 2000) observed the influence on pattern formation in the chromatograms of HP treated milk. Flags occurred to be less defined and thinner. At higher pressures (500 to 700 MPa) a rising inhibition occurred in the bowl zone. Wohlers (2011) studied the influences of different farming practices on the the outcomes of the Steigbild experiments. She also investigated the effects of heat treatment (100 °C,

boiling up of milk): She found the flags to be thinner and more parallel and more 'rigid'. The bowl zone was observed to be 'washed out' and less differentiated.

2.4.2 Circular chromatography

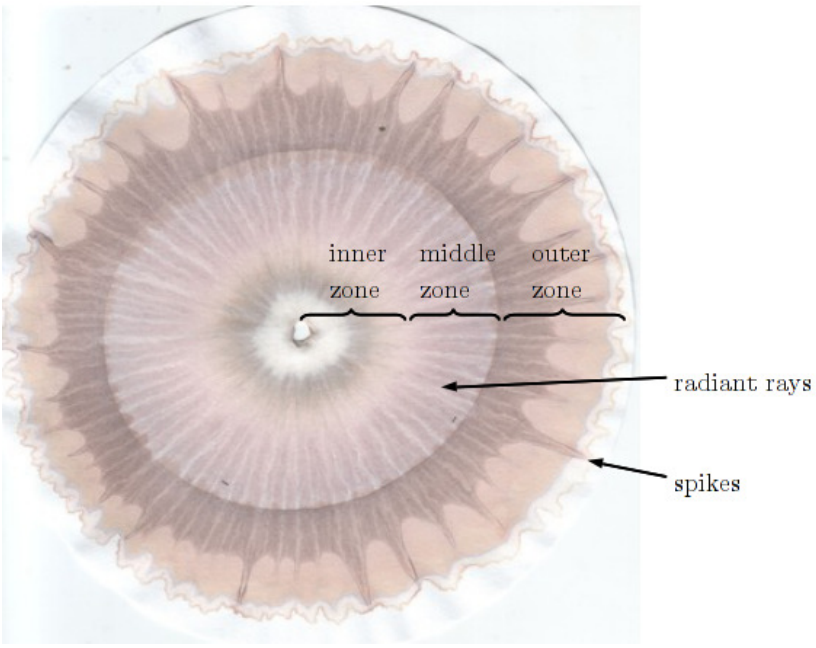


Figure 2.22: Circular chromatogram with three zones: inner zone, middle zone and outer zone.

Ehrenfried Pfeiffer, who pioneered the biocrystallization method (see below), also experimented with the rising picture method and in 1960, he developed an adapted version: the "Rundfilterchromatographie" (English: "circular chromatography") Pfeiffer (1984). Among others, the method was also developed to determine the quality of compost and soil samples, which Brinton Jr (1983); Pfeiffer (1984) studied in detail. They discovered that qualitative and quantitative conclusions can be drawn form the chromatograms, such as the humus condition of different soils. Knorr (1982) found a correlation between protein content of the extract (he used protein solutions and freeze dried collard plants) and the size of the inner zone to which Knorr refers as the "area of lowest density" (Knorr, 1982, p. 16). Others like, Beckmann et al. (1993); Fritz et al. (2009, 2011) applied the method as a simple and inexpensive testing tool for food quality and to show the influence of farming methods and soil quality on agriculture produce.

Methodology The methodology of the circular chromatographic method resembles the procedure of the Steigbild method, however the filter does not stand vertically in a dish, but the round filter paper is placed vertically on a petri-dish a watch glass containing either the food extract or a $AgNO_3$ solution, compare figure 3.7 on page 54.

A hole is punched through the middle of the filter, in which a filter paper wick is pushed that touches the liquid. Through capillary forces, the liquids rise upwards through the wick and spread horizontally into the filter. In the two-step procedure, the paper is preconditioned with a $AgNO_3$ solution up to a certain radius which is sample specific, followed by the food extract, made using a sodium hydroxide solution ($NaOH$) which spreads further on the paper. The chromatograms then dry under controlled atmosphere and diffuse light conditions. An example is shown in figure 2.22. For more information see (Knorr, 1982; Knorr and Vogtmann, 1983; Pfeiffer, 1984; Beckmann et al., 1993)

Picture evaluation As shown in figure 2.22, the chromatograms obtained consist of three different zones: An inner, middle and outer zone. The inner zone evolves around the center and is usually lightly coloured or blank. In the middle zone, differently coloured radiant rays occur. Depending on the sample, these rays can be darker (humus) or lighter. There can occur differently coloured rings, depending on the sample and its state. The middle zone reaches until the flow limit of the $AgNO_3$ solution. The outer zone shows the typical spikes and the most intense colours of the chromatogram. Within the valleys of the spikes a variation of coloured patterns can occur, like clouds or droplets or points. The radiant rays of the middle zone can reach until the tips of the spikes. Depending on the appearance of the three zones, the pictures are evaluated qualitatively. For example, among different samples, colours within the zones can vary or the appearance of the spikes in the outer zone can vary be different, for example vary in size or shape (Brinton Jr, 1983; Beckmann et al., 1993; Balzer-Graf and Gallmann, 2000). Unlike for the Steigbild method, no standardized visual picture analysis has been developed until now.

As mentioned above, Knorr (1982) suggested that the circular chromatographic method can also be applied for quantitative evaluation, as he found a linear correlation between the protein content of extracts made from collard plants and the size of the inner zone. Thus, by measuring the radius of the inner zone, the protein content of other food samples could potentially be determined via the circular chromatographic method.

Pattern formation According to Brinton Jr (1983), who studied the feasibility of the circular chromatographic method on the assessment of humus conditions, pattern formation of the chromatograms happens as follows: $AgNO_3$ reacts with the $NaOH$ to silver hydroxide ($AgOH$), which then almost immediately reacts further to silver oxide (Ag_2O):

$$AgNO_3 + NaOH \rightarrow AgOH + NaNO_3$$

$$2AgOH \rightarrow Ag_2O + H_2O$$

Ag_2O is insoluble and causes a yellow-brown participate. $AgOH$ on the other hand is weakly soluble and partially blocks the pores of the chromatographic paper. This results in a streaking effect, see figure 2.22. Large molecules like proteins from the extract enhance this effect. Knorr (1982) also discussed the possible reaction of $AgNO_3$ with certain amino acids.

Effects of milk treatments on pattern formation Likewise the Steigbild method, only a few authors attended to milk as a study object for circular chromatography. As mentioned above, it was mainly used before to assess the condition or quality of soils and plant material. Balzer-Graf and Gallmann (2000) researched the impact of HP treatment on milk. They found irregularities in the middle zones of the chromatograms of HP treated samples. Beckmann et al. (1993) observed changes in colour in different rings for high pasteurized milk (85 °C for 8-10 s). Furthermore, radiant rays seemed to be less distinct.

2.4.3 The biocrystallization method

The biocrystallization method was first developed by Ehrenfried Pfeiffer in 1930 who called it "empfindliche Kristallisation" or 'sensitive crystallization' (Pfeiffer, 1930 in Kahl, 2007). The term 'biocrystallization' was given by Magda Engquist (Engquist, 1970).

In the last years, several research groups developed this method further and validated it for certain research questions, such as the differentiation between food samples from organic and conventional produce, respectively (Kahl, 2007). The methodology was standardized including visual evaluation (Huber et al., 2010), texture analysis (Meelursarn, 2007) and structure analysis (Doesburg and Nierop, 2013). In the past, the method has also been used to detect diseases by using blood as a sample, compare Selawry and Selawry (1957).

Methodology For solid foods, a food extract is obtained, usually by chopping and followed pressing of the food then mixed with distilled water. Finally, the cupric chloride solution ($CuCl_2$) is added. For liquid foods, such as milk it is adequate to only mix the sample with water and $CuCl_2$. To obtain clearly structured pictures, the optimal concentrations of sample (extract) and $CuCl_2$ first have to be determined in pretests, otherwise the resulting pictures will not be usable for image analysis as they may show clear signs of over- or under-concentration (Engquist, 1970; Balzer-Graf and Balzer, 1991).

After mixing, 6 ml of the the mixture is pipetted on a float glass plate with a 9 cm diameter ring on it put together by Vaseline. Following a randomized order, the dishes are then put onto one of two rings inside a vibration-free crystallization chamber with

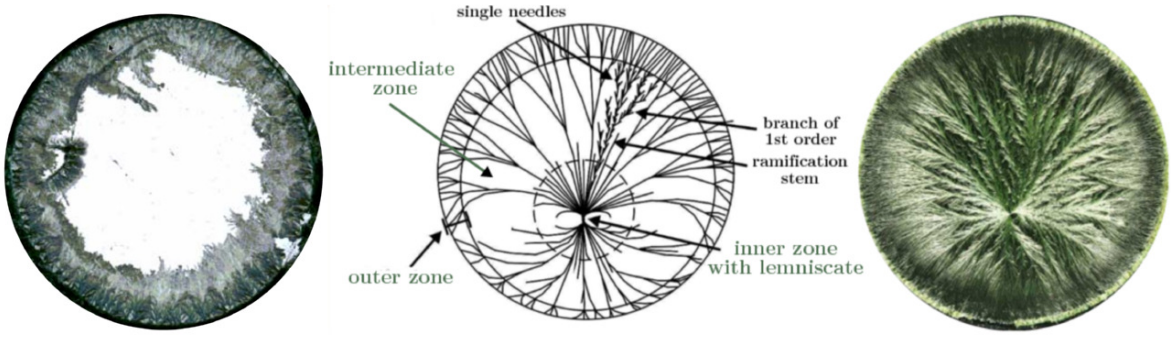


Figure 2.23: Biocrystallograms: a) chrystallogram derived from cupric chloride solution without the addition of a sample (Meelursarn, 2007, figure 2.1, p.5), b) scheme of a biocrystallization picture with some morphological criteria, c) biocystallogram from a fresh apple sample. (Doesburg et al., 2009 figure 4.4.1 on p. 7, and figure 18.3.1 on p. 45, respectively).

controlled humidity (53 % relative humidity) and temperature (30 °C) that stands in another climate controlled room (26 °C, same humidity), compare figure 3.9 on page 56. The two rings have room for 43 dishes, compare Kahl (2007) and appendix C. There, the mixture crystallizes after 10 to 16 hours due to the evaporation of water and the following over-saturation of the $CuCl_2$ solution. The crystallization process is monitored via a camera fixed to the ceiling of the climate chamber, which takes a photo every ten minutes.

The difficulties with this method are the sensitivity to outer influences such as vibrations and fluctuation in temperature and humidity. It has been shown that the forms of pictures with variant nucleation times[6] differ in their morphology. The most characteristic difference seen in late crystallized pictures (15-16 hours) is the formation of multiple centers. Early and 'middle' pictures usually show only one center, sometimes two. Thus, for image analysis these late crystallized pictures are usually sorted out.

The crystallogram and its patterns $CuCl_2$ solution alone forms patterns, which are characteristic for cupric chloride crystals (see figure 2.23 a)). With the added sample (extract) patterns or 'gestalts'[7] are formed consisting out of crystal stems, branches and needles. In figure 2.23 b) one can see a scheme of a typical biocrystallization picture. An example of a crystallized food extract can be seen in figure 2.23 c). It shows a photo made from a crystallized fresh apple sample. We can observe a clear center which indicates the point from where the crystallization starts and which forms a lemniscate pattern (∞). As the crystallization process goes on, so called ramification

[6] the time when the crystallization starts. It is monitored by a camera.

[7] The term 'gestalt' can be translated as 'shape' of 'form' based on Goethe's natural scientific works in which he refers to 'gestalt' as an expression of the essence (translated from German "Wesen") or nature of things (Steiner, 1990).

stems are formed in the intermediate zone from which branches divert on multiple levels, until the branches consist of just a single needle. The needles can be rather broad or fine and only visible with a magnifying glass. Crystallization stops in the outer zone, where only single needles are present. The forms or 'gestalts' are highly sample specific: for example pictures obtained from apples show a different 'gestalt' than pictures obtained from milk (Kahl, 2007).

Next to the concept of 'order' and 'life processes' that are supposed to be involved in pattern formation (see section 2.4), Selawry and Selawry (1957) tried to understand how the different patterns emerge on a more molecular level. They linked it to the different proteins present in the samples that are responsible for the differences in crystal formation and angles of branches and needles. However, in the 1950s, possibilities for more precise analytics did not exist. They guessed that also the presence of trace compounds might have an influence on pattern formation.

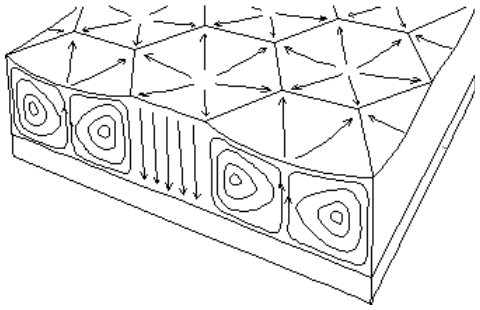


Figure 2.24: Flow pattern within the Bénard-cells (Velard and Normand, 1980).

Busscher et al. (2006) proposed that the evolving of the typical crystallization structure with one nucleation point follows a process of self-organization due to instabilities in the liquid as an open system. His theory is derived from the self-organizing processes (Bénard-Maragoni-phenomenon) in liquids heated from below leading to the co called Bénard-cells, see figure 2.24. When the liquid gets heated from below, the temperature difference between bottom and surface of the liquid becomes bigger. At low temperature gradients, the heat gets transferred through the liquid via heat conduction[8]. When the temperature gradient crosses a critical point, heat transport changes to convection[9] and a suction is created, which leads to the formation of the cells shown in figure 2.24. For more details about this phenomenon see for example Velard and Normand (1980). In the case of the biocrystallograms, gradients of surface-tension and $CuCl_2$ concentration occur during evaporation of the water as evaporation happens faster around the middle of the plate. These gradients could lead to the formation of a Bénard cell. Nucleation starts in the center and stops at the boundaries of the cell. For plates with multiple centers, several Bénard-cells could have been formed, this usually

[8]heat conduction is the transport of heat due to a temperature gradient via diffusion and the collision of particles.

[9]convection is the collective movement of molecules in a fluid, for example a liquid.

happens at delayed crystallization. However, the process of formation of the different 'gestalts' is not explained by this theory.

Picture evaluation Today, visual evaluation of the patterns is at an advanced stage. The pictures are interpreted visually after morphological criteria, such as 'Durchstrahlung' or 'Quernadeln', "based on ranking/scoring of discrete scales of various morphological features in the macro- and microscopic crystal structure. One feature for visual evaluation is the coordination of the crystal structure, i.e., the degree of [sic.] which the individual crystals are randomly or orderly distributed over the glass plate" (Meelursarn, 2007, p. 2). The evaluation also includes comparing the samples to a reference scale consisting of pictures from previous experiments if existent. Visual evaluation happens according to adapted ISO-norms from sensory analysis. Much training and experience is required to be able to identify morphological criteria and to distinguish fine variations in the images obtained from different samples.

Two other recently developed tools are computerized texture and structure analysis. With these tools, a very objective statistical analysis of several different parameters can be performed. Hereby, for texture analysis, the scanned pictures are converted to grey-scale images. The texture analysis program then calculates the differences in grey-scale level of the neighbouring pixels over certain regions of interest (ROI). With ROI, a circular area around the nucleation center[10] is meant. It can range between 0 and 100 % of the plate area, 0 % representing the nucleation center and 100 % representing the entire plate, from center to edge. Structure analysis focuses on the length and broadness of the needles. With all these tools developed for image analysis, the biocrystallization method is best developed and standardized picture forming method today. (Kahl, 2007; Meelursarn, 2007; Doesburg and Nierop, 2013)

Effects of milk treatments on pattern formation In the last decade(s) the biocrystallization method has been used to assess a large number of research questions, for example the comparison and differentiation between organic and conventional food stuffs, different soil types, planting distances and varieties or the influence of processing techniques such as heat on the food, see for example Balzer-Graf and Gallmann (2000); Andersen et al. (2001); Knijpenga (2001); Kahl (2007); Kahl et al. (2008, 2009).

Only recently, also the impact of treatments on milk was investigated by several authors. Balzer-Graf and Gallmann (2000) studied the influence of HP on pattern formation: Crystallograms of HP treated samples showed broader branching in the outer zone and needles seemed to be thinner. Wohlers et al. (2007) described the crystallization patterns of raw and UHT treated milk. Also here, an increased amount of thinner needles was observed. Branches seemed to be more bent for the UHT treated samples. The outer zone of the crystallograms appeared more clear. Furthermore,

[10] the point where the crystallization starts

Kahl et al. (2009) found impacts of homogenization on the crystal patterns: they state that "the crystallization structure loses complexity when the milk sample undergoes homogenization" (p. 176). With texture analysis, they detected a significant difference between raw and homogenized milk ($p < 0.01$ for ROI < 80 %).

3 Materials and methods

In this chapter, the materials and methods used in this thesis are presented. An emphasis was given on the methodology of the PFMs as they are generally less known. All experiments were performed in repetition for each treatment to check the reliability of the standard analytical as well as the PFMs.

3.1 Milk samples

The raw milk samples were obtained from the German Federal Institute for Risk Assessment in Berlin. The milk was collected from a herd of ten to twelve lactating cows which all belonged to the Holstein breed[1]. The cows were fed with a mixture of maize silage, lucerne hay, shredded rapeseed, shredded soy extract[2], straw, beets, sugar beet chips and complemental fodder. For the samples, evening milk of one day and morning milk of the other day were collected, mixed and filled in 2 liter plastic bottles. The milk was picked up directly after milking on the morning of the second day. The transport to the Department of Food Biotechnology and Food Process Engineering happened under cooled conditions in a cool box to avoid rising of temperature above + 8 °C and therefore prevent increased microbial growth.

3.2 Treatments

Four different types of treatments were performed on the milk samples: heat treatment, skimming, HP treatment and PEF treatment. As we saw before in section 2.2, two treatments (skimming and PEF) were chosen because they are said to not cause denaturation of the milk proteins. The other two treatments (heat and HP) were chosen because they are said to cause protein denaturation, however in different ways. For each treatment besides skimming, two intensities were chosen: a milder and a higher intensity.

3.2.1 Heat treatment

Milk was heated at 75 °C for 15s and at 85 °C for 5min.

For the mild treatment (75 °C 15 s), cooled milk (+ 4-8 °C) was poured into a beaker. With a tube pump (Watson Marlow 323, Watson Marlow Bredel Pump,

[1] a breed known for its very high milk production rates
[2] from genetically modified soy

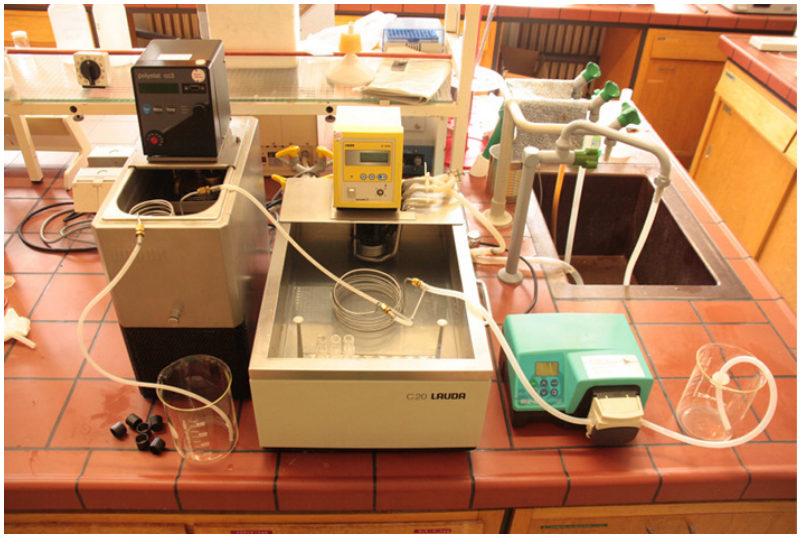


Figure 3.1: System used for the 85 °C heat treatment consisting of a silicone oil bath, a water bath and, a tube pump and a system of silicone and metal tubes (described and explained in the text).

Falmouth, USA) and flexible silicone tube (6 mm inner Ø [diameter]), the milk was pumped into a 78 °C warm water bath (Ecoline Staredition E 100, Lauda, Lauda-Königshofen, GER) at 70 rpm speed and a flow rate of $\dot{V}$ =8.4 l/h (or 2.33 ml/s). In the water bath, the milk was transfered through a metal helix tube (1 mm inner Ø) to preheat the sample up to 73.0 °C). Consequently, the milk was piped through another silicone tube (3 mm inner Ø) into a bath filled with silicone oil (Polystat K8, Huber Kältemaschinen GmbH, Offenburg, GER; control unit: Polystat cc3, same producer) containing another metal helix cube, with a temperature of 78 °C to bring the sample to the final temperature of 75.0 °C. Finally, another silicone tube of 6 mm inner diameter was led back into and out of the water bath of 75 °C to collect the samples in thin 10 ml glass test tubes placed in ice to cool them down rapidly. Lids were placed on the test tubes. The dwell time in the last silicone tube was taken as the holding time τ_h and was set to 15 s via calculating the length l of the tube:

$$\tau_h = \frac{V_t}{\dot{V}} = \frac{\Pi r^2 \cdot l}{\dot{V}} \rightarrow l = \frac{\tau \cdot \dot{V}}{\Pi r^2} = 124.0\, cm$$

whereas V_t describes the volume of the final silicone tube and r its radius.

The temperature of the milk at the outlet of each heating bath was measured via a digital thermometer (Testo 110, Testo, Alton, UK).

For the intense treatment (85 °C, 5 min), the installation was slightly altered, differing in the length and thickness of the last silicone tube (3 mm inner Ø and 40 cm length) and the set temperatures of the heating baths (water bath: 85 °C, measured outlet-T: 82.7 °C and silicone bath: 88 °C, outlet temperature: 85.0 °C). The last silicone tube was not led through the water bath again but directly into the glass test

tubes that were placed in the water bath with the aid of a test tube rack, see figure 3.1. The test tubes were closed with lids to prevent water evaporation. Time measurement was started as soon as the heated milk entered the first glass test tube. After 5 min, the test tubes were taken out of the water bath and put on ice. For each of the two experiments, four glass test tubes with 10 ml heat treated milk each were obtained.

3.2.2 Skimming

The skimming of the milk was performed using a centrifuge suitable for 50 ml centrifuge tubes (Megafuge 1.0R, Thermo Scientific, Waltham, USA). 50 ml of the 6-8 °C cold raw milk sample was poured in each of two centrifuge tubes and closed with a lid. The tubes were centrifuged at 2000 g for 20 min at 20 °C. Thereafter, the tubes were taken out, the lids were opened and a piece of fat was taken off the samples with the aid of a spatula. Through the hole created in the fat-layer, the milk was poured into another centrifuge tube through a folded filter (Whatman 595 1/2 90 mm Ø, Whatman, Dassel, GER), placed in a funnel. The obtained skimmed milk samples were stored at 6-8 °C until analysis.

3.2.3 High pressure treatment

For the HP treatment, 25 ml of the cooled raw milk was poured into a polyethylene (PE) bag and shrink-wrapped under slight vacuum conditions. The PE-bags remain flexible under HP-conditions. Afterwards, the samples were put into another bag and shrink-wrapped again under the same vacuum conditions.

The two bags were put into the vessel of the HP-unit (single vessel U4000, Unipress Equipment Division, Warsaw, POL), shown in figure 3.2 which was filled with a 1:1 (v/v) mixture of 1-2-propanediol and water. Two subsamples each were treated simultaneously at 300 MPa and 600 MPa, respectively for 10 min at room temperature. Afterwards, the samples were taken out, poured into a centrifuge tube and stored at 6-8 °C until analysis.

3.2.4 Pulsed electric fields treatment

PEF treatment was performed using a high intensity PEF unit, which was designed and build by the Department of Food Biotechnology and Food Process Engineering. Mayor components were the high voltage generator (Lambda-Emi, Neptune, USA), a frequency generator (Thurbly Thandar Instruments, Huntington, UK), a 1 μF energy storage capacitor, a spark gap switch and a parallel plate treatment chamber (size of gap and plate size: 1 cm x 25.2 cm x 8.2 cm [w x l x h]) for batch treatment.

162 g (m) of cold milk (6-8 °C) was poured into the treatment chamber. Temperature was measured before and after treatment with a digital thermometer (Testo 110,

Figure 3.2: HP-unit single Vessel U4000, closed.

a) b) c) and d) e)

Figure 3.3: PEF-unit: a) view on the entire unit, b) treatment chamber, c) (above image) frequency generator (top) and power supply (below image), d) (bottom) parallel plate treatment chamber and e) connected parallel plate treatment chamber (without sample).

Testo, Alton, UK). Conductivity was determined using a TatraCon 325 conductometer (WTW, Weilheim, GER) Total energy input was chosen to be 45 kJ/kg for the milder treatment and 116 kJ/kg for the more intense treatment, with a field strength E of 10 kV/cm for both of the treatments, a capacitance C of 1 μF and exponentially decaying pulses with a pulse width τ of 50 μs. The specific energy per pulse W_{pulse} was calculated via the following formula

$$W_{pulse} = \frac{1}{2} U^2 \cdot C$$

with U being the voltage between the two electrodes. U was calculated as

$$U = E \cdot d = 10 \frac{kV}{cm} \cdot 1\,cm = 10\,kV$$

The specific energy per pulse W_{pulse} was therefore 50 J.

Knowing W_{pulse}, the number of pulses n could be calculated that are necessary to obtain the desired energy inputs:

$$E = \frac{W_{pulse} \cdot n}{m}$$

and so

$$n = \frac{E \cdot m}{W_{pulse}}$$

with the outcome of 146 pulses for 45 kJ/kg and 376 pulses for 116 kJ/kg energy input, respectively. Each of the the treatments were performed in repetition.

After the treatment, the milk was poured into centrifuge tubes and stored at 6-8 °C until analysis.

3.3 Standard analytical methods

The standard analytical methods chosen for the experiments of this thesis include RP-HPLC for the detection of structural changes in the protein fraction, especially denaturation of caseins and whey proteins, the Bradford-method for the determination of the total protein content and the Röse-Gottlieb-method as a tool to identify the fat content of the milk samples.

3.3.1 Determination of the grade of milk protein denaturation (HPLC)

The following description of the HPLC-based identification of treatment induced changes of milk protein fractions follows the summary given in the thesis of Schiller (2013), undertaken at the Department of Food Biotechnology and Food Process Engineering.

First sample preparations After the samples were submitted to one of the above mentioned treatments heat, PEF or HP, respectively, the samples were prepared for HPLC-analysis. For this, about 10 ml of the cooled samples was taken out of the fridge, poured into new centrifuge tubes and let stand for some time until room temperature was reached. In the following, the samples were prepared in three different ways:

1. the samples were directly submitted to further preparation (see below). These samples will be called *control sample* in the following and serve as a control to preclude losses of water or protein in the samples caused by the treatment.

2. approximately 1.5 ml of the samples was poured into a small 2 ml test tube suitable for centrifugation. Samples were centrifuged at 10,000 g for 30 min at room temperature with an Eppendorf Centrifuge 5804R (Wesseling-Berzdorf, GER). The supernatant was collected with a pasteur pipette at a hight of 1cm

measured from the bottom of the tube and transfered into another test tube. Subsequently, the samples were submitted to further preparation. In the following, these samples will be called *centrifuged samples*. With these samples, one can draw conclusions about the state of proteins in the serum phase, especially about structural changes of the caseins.

3. In a 20 ml glass tube, 5 ml of each sample was diluted in 12.5 ml of distilled water to a protein concentration of approximately 1%. Then, the pH was adjusted to pH 4.6 (at the caseins' isoelectric point) with the aid of a 1 molar solution of hydrochloric acid under continuous stirring to precipitate the caseins plus potentially attached whey proteins. After that, approximately 1.5 ml were poured into a test tube and followed the preparation of the *centrifuged* samples. With these samples structural changes and in particular the denaturation of the whey proteins can be studied. These samples will be called *pH-samples* in the following.

Following sample preparation The subsequent sample preparation followed in general the description for purified protein in Bordin et al. (2001), with some slight changes.

A solution consisting of 91.8 mg of sodium citrate tribasic dihydrate , 192.8 mg of DL-dithiotreitol (both $\geq$ 99.0% purity, purchased from Sigma Aldrich Chemie GmbH, Steinheim, GER) and 35.825 g of guanidine-hydrochloride (purchased from $\geq$98.0% purity, Alfa Aesar GmbH & Co KG, Karlsruhe, GER) were weighed out and dissolved in water under stirring and slight heating. The pH of this solution was adjusted to a value of 7.0 with the aid of a 1 molar sodium hydroxide solution and filled up to 50 ml with distillated water. The solution was kept under cooled conditions up to about four weeks.

The samples that underwent the first sample preparations, were now diluted with the above solution of guanidine at the ratio of 1:5 (180 μl : 720 μl) in a test tube. The mixtures were then heat treated at 60 °C for 10 min in a thermo-shaker (MKR 13, HLC by DITABIS, Pforzheim, Germany). Hereby the guanidine acts as a cheotrope substance. This leads to an unfolding of the proteins in the sample which cannot keep up their folded state any longer (Römpp and Falbe, 1999). Heating accelerated the unfolding. Afterwards, the samples were let cool down to room temperature and diluted with distillated water in a centrifuge tube at the ratio of 1:4 (750 μl : 2250 μl). Thereafter, the prepared samples were filtered through a 25 mm Ø membrane syringe filter made out of polytetrafluoroethylene (PTFE) with a pore size of 0.45 μm (VWR International LLC, Radnor, USA). The filtrate was gathered in vials. The big filter diameter was chosen to reduce the filter pressure so that any remaining fat in the sample would be filtered out and not been pressed through the membrane as the fat might cause unwanted side effects in the HPLC-measurement and clog the column.

HPLC-analysis The settings for HPLC-analysis were mainly taken from Bordin et al. (2001), with slight changes.

The HPLC unit (Dionex UltiMate 3000, Thermo Fisher Scientific, Sunnyvale, USA) shown in figure 3.4 contains a low-pressure gradient pump (LPG-3000SD) with integrated vacuum degasser, a capillary mixer of 50 μl and a static mixer of 350 μl volume, respectively. The different eluents are mixed in the two latter. The auto-sampler (WPS-3000SL, without thermostatting option) contains a syringe, a sample loop and a buffer loop of 100 μl volume each. The DAD used here has a flow cell volume of 13 μl and an optical path length of 10 mm. The detector is able to record a spectrum additional to the regular DAD for each detected peak. The measuring was carried out in the range of UV-light at 214 nm wavelength.

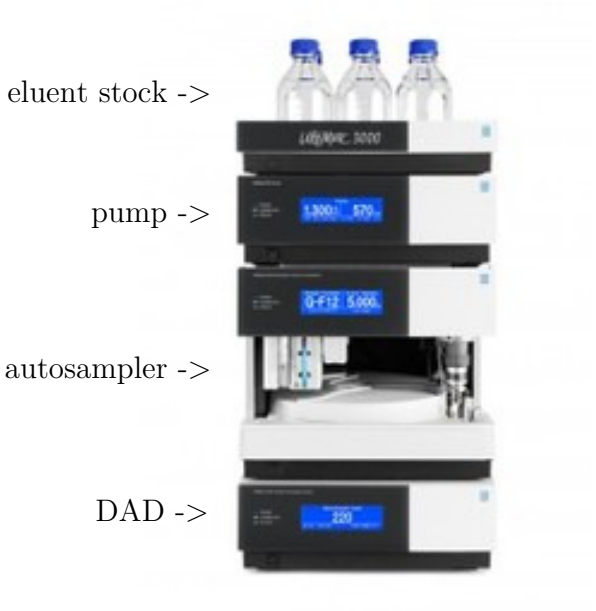


Figure 3.4: HPLC-unit with eluent stock, low-pressure gradient pump, auto-sampler and DAD.

A reversed phase C4-separating column (Jupiter 5u, Phenomenex, Torrance, USA) was used for analysis. The column had a particle size of 5 μm and a pore size of 300 Å. The column was 150 mm long and had a diameter of 2.0 mm. The filling material was modified silica gel. In front of the column, a guard cartridge of 4mm length and a 2 mm Ø was installed to protect the column additionally. The temperature of the column was set to 40 °C.

The two eluents for the analysis were

eluent A milli-Q (high-purity) water with 2 ml of trifluoroacetic acid (TFA, Merck Schuchardt OHG, Hohenbrunn, GER) per liter

eluent B acetonitrile (Rotisolv® HPLC Ultra Gradient, Carl Roth GmbH & Co. KG, Karlsruhe, GER) with 1.7 ml of TFA per liter.

The gradient elution was conducted after the following scheme as seen in table 3.1.

Table 3.1: Scheme of the gradient elution for HPLC-analysis

time [min]	eluent A [%]	eluent B [%]
0	70	30
16	59	41
33	54.9	45.1

Six different milk protein fractions were determined (in order of their retention time):

caseins κ-casein, α-casein and β-casein

whey proteins β-lactoglobulin B, α-lactalbumin and β-lactoglobulin A

The single proteins were determined with the aid of protein standards, as shown in a previous thesis submitted by Schiller (2013). The concentration of the single protein fractions could have been determined by the aid of a calibration. However, this was not done within the framework of this thesis, but rather another method was used (see below in section 3.3.2). For the evaluation, the concentrations were determined indirectly by putting them in relation with the original sample. The detected areas of the single proteins were put into relation with the particular injection volume. For the pH-samples, the dilution was calculated in as well. Subsequently, the obtained areas were put in relation to the original sample which was taken as 100%. Due to poor protein separation between the whey proteins β-lactoglobulin B and α-lactalbumin, and the mostly uniform behaviour of the different casein fractions, the results of the different casein and whey protein fractions were combined.

3.3.2 Determination of the protein content (Bradford)

As mentioned above in section 3.3.1, the protein content of the samples was determined using the Bradford method. The Bradford-method was chosen because it is a highly specific method and not susceptible to errors caused by other milk components such as fat particles.

The raw milk samples were diluted 1:400 with distillated water (in small steps of 1:4 $\rightarrow$ 1:40 $\rightarrow$ 1:400) in triplicates. Only untreated milk was used to investigate the total protein content. The Bradford reagent (Bio-Rad Protein Assay, Bio-Rad Laboratories GmbH, München, GER) was diluted at a ratio of 1:4.6 with distillated water and then filtered through a Whatman #1 filter. 100 μl of sample was pipetted to 1150 μl of diluted Bradford reagent. After an incubation of precisely 15 min, the absorption of the samples was measured with an UV/Vis-spectro-photometer at an excitation wavelength of 595 nm in semi-micro plastic cuvettes (VWR). Water plus diluted Bradford reagent

functioned as a blank value. All diluted samples were analyzed in triplicates, so each raw milk sample was tested for protein content nine-fold.

Beforehand, a linear calibration curve with defined concentrations of bovine serum albumin (BSA, ≥96 % purity, Sigma Aldrich) was generated, with BSA-concentrations varying from 10 to 100 μg/ml (see appendix B). With the help of this linear calibration curve, a correlation between absorption and protein concentration can be drawn. Milk protein concentrations within the range of 0.4 to 4.0 % can be detected. As the natural protein content of bovine milk generally ranges between 2.8 and 3.6 % (Töpel, 2004), all milk protein concentrations lay in the linear range of the calibration curve.

With the help of the total protein concentration and the HPLC results of the single proteins (total detected area and areas of single proteins), the protein concentrations for every protein fraction could be determined.

3.3.3 Determination of the fat content (Röse-Gottlieb)

The fat contents of the raw and skimmed milk samples were determined with the Röse-Gottlieb method, according to DIN EN ISO 1211:2010-11 with one slight change: instead of diethyl ether, tert-butyl methyl ether (tBME, SupraSolv, Merck) was used for extraction as it is less flammable than diethylether. The samples were analyzed at least in duplicate with distillated water as a blind value. Besides tBME, the other following chemicals were used for analysis: ammonia solution 25% (Merck), petroleum benzine boiling range 40-60 °C (Merck), ethanol purum 96% without additives (Sigma Aldrich).

3.4 Picture forming methods

In this section, the methodology of the three chosen PFMs Steigbild, circular chromatography and biocrystallization are presented. Furthermore, the different and tools used for the evaluation of the obtained chromatograms and crystallograms are explained. All experiments were performed twice with different samples for each treatment to check the reliability of the methods.

3.4.1 Steigbild

The methodology for the Steigbild experiments was based on the PhD-thesis of Wohlers (2011).

Preparations Fifteen numbered filter papers of the type 2043A, a size of 170x170 mm with a cutout of 24x125 mm were rolled to cylinders at least one week before the experiments. They were covered with paper and fixed with paper clips. At least one day before the experiment, the roll was opened and the filter papers were folded

individually and closed with a paper clip to make the fit exactly in the Kaelin-dish (with a diameter of 7 cm and a furrow of 0.5 cm depth, invented for the Steigbild method by W. Kaelin, 1965), see figure 3.5 b). This was reached when the bottom edge of the filter paper reaches the lowest part of the furrow. Only then, a homogeneous rising of the liquids can be guaranteed. The filter papers were touched exclusively with gloves during the whole procedure.

a) b)

Figure 3.5: a) climate cabinet used for the Steigbild experiments, b) prepared filter standing in Kaelin-dish.

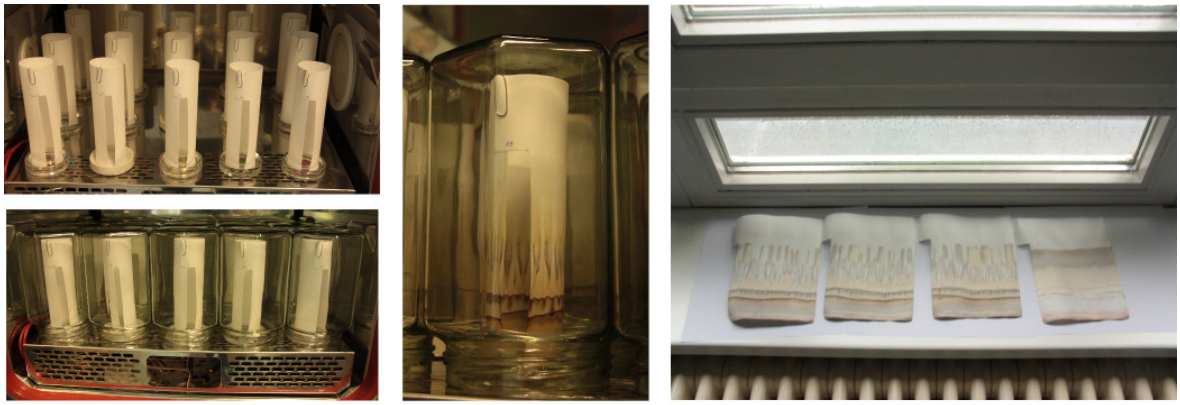
The Steigbild experiments were performed in a climate cabinet (WK1 180/40, Weiss Umwelttechnik GmbH, Reiskirchen-Lindenstruth, GER) shown in figure 3.5 a). Unfortunately, it was not possible to regulate the air flow of the ventilator in the climate cabinet. To protect the filter papers from being blown away by the ventilator air flow, a board was placed below the ventilator. However, air mixing was still guaranteed due to gaps between the inner surface of the climate cabinet and the board as well as slits inside the board. The samples were protected from light by placing light reflecting foil before the window of the climate cabinet. Additionally, the lights inside the climate cabinet were switched off during the whole procedure.

The cooled raw and treated milk samples were diluted 1:7 with destillated water. Sample preparation was carried out in duplicates.

The Kaelin dishes were placed inside the climate chamber in three rows of five (see figure 3.6 a)). Samples were distributed in a randomized, but fixed order. Colour coding dots were placed on the lower board to mark the different samples and subsamples. Out of the fifteen dishes, four were used for each of the three sample preparations (untreated, treated with mild and high intensity), from which two for each subsample. The remaining three dishes were used for the analysis of water as a standard.

0.25% (w/w) watery solutions of silver nitrate ($AgNO_3$, 99.8% purity, Merck) and iron(II)sulfate heptahydrate ($FeSO_4$, Merck) with a total volume of 50 ml were

prepared. The solutions was used for analysis within a period of two weeks.



a) and b) c) d)

Figure 3.6: Set up for the Steigbild experiments: a) (above) first rising phase, b) (below) second rising phase with glasses put over the filters, c) end of third rising phase, d) drying and picture developing in diffuse light conditions.

Rising phases and picture developing

1. For the first rising phase, 0.6 ml of prepared sample was pipetted into a Kaelin dish. Thereafter the Kaelin dish was slewed with circular movements to ensure homogeneous distribution of the sample dilution. To guarantee the absence of air bubbles that might affect the rising of the liquids, the filter papers were placed in the dishes with a circular movement. Hereby the label (remainder of the cutout) was located at the top. The climate cabinet was closed, temperature was set to 20 °C and relative humidity to 60 %. The filter papers remained in the chamber for three hours. Figure 3.6 a) shows the filters at the beginning of the first rising phase.

2. Thereafter, the filter papers were taken out. With the aid of a stiff cardboard roll, a set square and a pencil, marks were set on the two vertical edges of the filter papers at a hight of 1 cm above the flow limit of the risen sample. The filters were then stored briefly upside down in a clean carton while 0.7 ml of the prepared $AgNO_3$ solution was pipetted in each dish and slewed for the second rising phase. The filter papers were placed in the dishes like described above and in the same order. After two filter papers were placed, hexagonal glasses ("preserving jar", height: 197.3 mm, diameter: 110,9/124,4 mm, volume: 1700 ml) were placed over the filter papers to rise humidity. Then the next two filter papers were placed and so forth until all filter papers were covered with glasses, see figure 3.6 b). The filters were let stand in the dark closed climate chamber for about fifteen minutes. The height of rise was checked with a small torch that was switched on only very briefly. After the $AgNO_3$ solution had risen up to the mark drawn

before, the glasses were taken off and the samples were left to dry for three hours in the same conditions as mentioned above.

3. Afterwards, the filter papers were taken out again and put into a closable carton to protect the filter papers from early light exposure. For the third and last rising phase, 2 ml of $FeSO_4$ solution was pipetted into the dishes. Further procedures were the same as for the second rising phase. After the $FeSO_4$ had reached the height of the lower edge of the label shown in figure 3.6 c), the glasses were taken off and the samples were let dry over night at 20 °C and 60 % relative humidity.

4. The next morning, the filter papers were taken out of the climate cabinet. They were straightened carefully on the edge of the laboratory bench, protected by a paper sheet on both sides. Then they were exposed to diffuse daylight conditions for six hours by putting them on a window sill on the north side of the building, see figure 3.6 d). After six hours, the filter papers were collected and labeled with date, experiment, sample and subsample.

Scanning On the same day, the filter papers were scanned with a flatbed scanner (Canon LiDE 30) without automatic colour correction at 600 dpi, 24 bit RGB colour and a G-value of 1.0. The pictures were saved in the .tiff format to prevent data loss. Although the scanner showed a shift in colour value towards red, no colour correction was used as the focus in the evaluation is on the differences between the pictures and was not on the highly accurate image representation. For the comparison, the colour shift was taken as acceptable.

3.4.2 Circular chromatography

The methodology for the circular chromatography experiments were based on the works of Brinton Jr (1983); Beckmann et al. (1993).

Preparation Circular filters of the type 2043A with a diameter of 150 mm (Schleicher & Schüll, Dassel, D) were used for the experiments. In each of the filters, a hole of 2 mm diameter size was punched into the center of the circle with a revolving hole punch (Knipex 90 70 220). On one side, the filters were marked with a pencil at a distance of four centimeters from the center. This was done twice at an angle of 90°. Of the same filter paper, 2x2 cm big pieces of filter paper were cut out carefully with the aid of a cutter knife and a set square. The squares were rolled up into thin paper wicks. Filters were touched exclusively with gloves during the whole experiment.

A 0.75 % (w/w) watery solutions of silver nitrate ($AgNO_3$, 99.8% purity, Merck) with a total volume of 50 ml was prepared. The solution was used for analysis within a period of two weeks.

1 ml of each cooled subsample was given to 9 ml of a 1% solution of $NaOH$. The samples were extracted during 1.5 hours. After that, the extractions were filtered through a folded filter (Whatman 595½, Whatman, Dassel, D) as flocculation of the fat took place during the extraction.

For the experiments, the same climate cabinet as for the Steigbild experiments was used, however with one additional grate, so that 16 chromatograms could be obtained at the same time (eight on each grate). Like with the Steigbild experiments, samples were distributed in a randomized, but fixed order. Colour coding dots were placed on both grates to mark the different samples and subsamples. The bottom parts of petri dishes (94 mm Ø, height: 16 mm) were placed inside the cabinet.

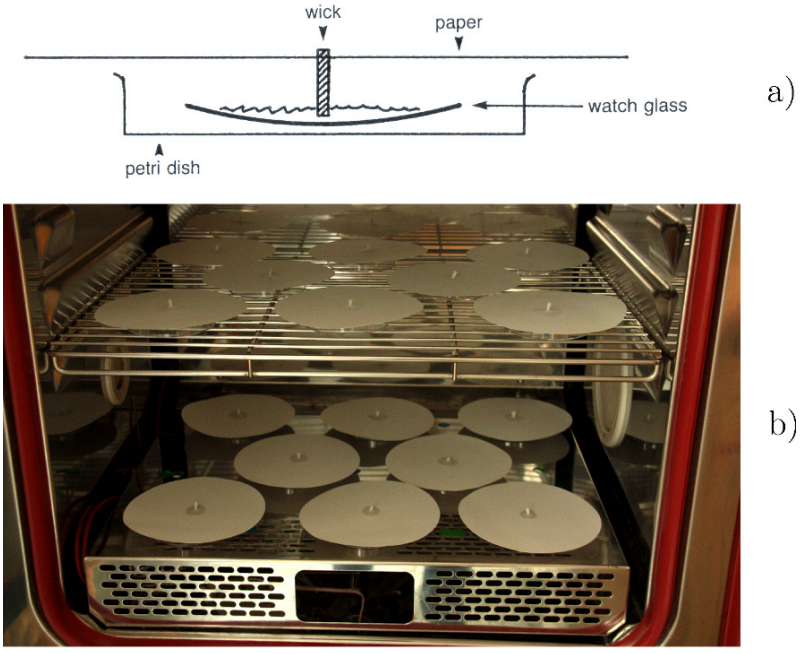


Figure 3.7: Set up for circular chromatography: a) filter paper and wick on petri dish with watch glass (taken from Brinton Jr, 1983, b) sensitizing of the filter papers with a $AgNO_3$ solution inside the climate cabinet.

Elution and developing

1. To sensitize the chromatic paper, 1.5 ml of the $AgNO_3$ solution were pipetted in each of 16 watch glasses with a diameter of 3 cm. The watch glasses were placed in the middle the petri dishes in the cabinet. Paper wicks were pushed carefully through the hole the middle of the prepared circular filters, in a way that the wicks touched the edges of the holes on all sides (see figure 3.7 a)). The filters plus wick were placed on top of the petri-dishes with the marked side up. Attention was paid to the correct placing of the filters: the wick had to stand upright and touch the bottom of the watch glass for correct and homogeneous running of the solution (see figure 3.7 b)). The door was closed to prevent early

development. After about 10 min, the solution had run until the 4 cm marks. At this point, the filters were taken out carefully and the wick was removed. Filters were laid out on and covered with non reactive white paper and put into a drawer for drying and to shield them from light. Maximum three layers of filters and paper were stacked on top of each other. The filters were let dry for one hour.

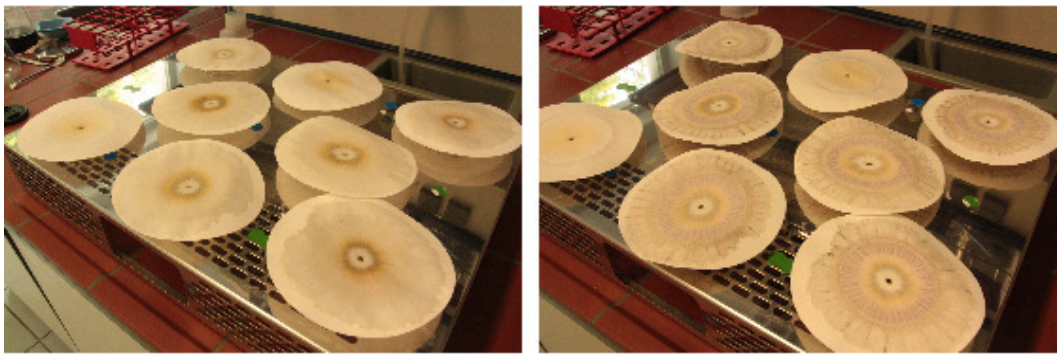


Figure 3.8: Developing of the circular chromatograms: a) start of development, b) developed chromatograms.

2. 2 ml of the sample extracts was pipetted into new watch glasses of 5 cm diameter which were then placed into the petri dishes inside the cabinet. Each subsample was replicated twice, giving four watch glasses and therefore chromatograms per sample (untreated, treated with low and high intensity, respectively). The dried filters were taken out of the drawer. Another paper wick was pushed carefully through the whole without damaging the filter and then put on top of the petri dish. The same care as above was spent on placing the filter and wick. Temperature was set to 23 °C with a relative humidity of 80 %.

3. When the extract was totally absorbed by the wick, after about 1 to 1½ hours, the grates were taken out of the cabinet and exposed to diffuse light. The wicks were taken out carefully again and the watch glasses removed. The filters were exposed to light for three hours until the colours were fully developed, see figure 3.8. Filters were labeled on the back edge with the date, experiment, sample and subsample.

Scanning Scanning was performed on the same day and in the same way as the Steigbild pictures.

3.4.3 Biocrystallization

The biocrystallization experiments were performed at the Crystal Lab in Ottersum, the Netherlands. Sample treatment, however, happened at the Department of Food

Biotechnology and Food Process Engineering in Berlin. Unlike the procedures with the Steigbild and circular chromatography, the milk was only treated with the highest intensity for each treatment. Milk of two different treatments plus untreated milk was used for each biocrystallization experiment (heat + PEF and skimming + HP). Depending on the experiment, 20 to 50 ml per subsample in centrifuge tubes were used with two repetitions per treatment. The samples were transported by train in a cool box to assure that the temperature of the milk would not rise above +8 °C. Beforehand, bronopol (≥98 % purity, Sigma) with a total concentration in the milk of 0.05 % (w/v) was added to prevent microbial growth and spoiling of the samples.

Preparations Sample preparation was conducted according to Kahl et al. (2009). The crystallization took place in a specially built climate controlled wooden chamber as described in Kahl (2007) placed in another climate controlled room. 43 dishes aligned in two rings were crystallized in one experiment. Climate conditions outside of the chamber were 26 °C and 53 % relative humidity, inside the chamber (over the plates) the temperature was 30 °C with the same humidity as outside. Temperature and humidity were checked on four places in both chambers, see figure 3.9.

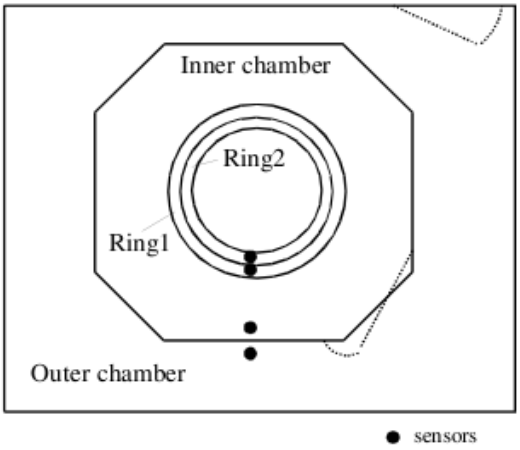


Figure 3.9: Scheme of the crystallization chamber with two rings and four sensors for detection of temperature and humidity (Kahl, 2007, p. 50) .

For the cupric chloride solution, $CuCl_2 \cdot 2H_2O$ (Merck), was dissolved in de-ionized water at a concentration of 10 % (w/w). Float glasses (diameter 100 mm, thickness: 2 mm; Pfähler, Gengenbach, GER) were cleaned using a laboratory washing machine (Miele), Neodisher Laboclean FLA and Neodisher Z (both Weigert, Hamburg, GER) at 90 °C with de-ionised water. After that, the glass plates were rinsed using 96 % ethanol (with 1 % methylethylketone [MEK], Roth) at let dry at room temperature. The plates were attached to 3.5 cm high acrylic rings (Broennum Plast, Rødovre, DK) using heated Vaseline. 43 dishes were placed into the chamber divided in two rings (inner and outer ring).

As a blank value or standard, an extraction of freeze dried wheat (Bio-Premium, Naturland, Meyermühle, Landhut, GER) was used: A 10 % (w/w) suspension of wheat with de-ionized, 25 °C warm water was prepared and extracted for 30min by constant stirring (200 rpm, horizontal shaker, Unimax 2010, Heidolph, Schwabach, GER) and then let stand for 15 min. 50 ml of the extract taken from 2 cm under the surface were filtered twice (Whatman 41 and 40). 9 ml of the $CuCl_2$ solution and 9 ml of the wheat extract were added to 42 ml of de-ionized water.

The centrifuge tubes containing the milk samples were gently moved in a circular and horizontal manner to homogenize the samples as some creaming took place during the transport. 10 ml of cooled milk sample was transferred into a 50 ml beaker which was left to stand for at least 30 min in a water bath, until 20 °C were reached. The rest of the samples were stored under cool conditions for further experiments. 2 ml of each sample were mixed with 43 ml of de-ionized water and 15 ml of the $CuCl_2$ solution and stirred at 120 rpm for 30 min (Heidolph horizontal shaker), see figure 3.10 a)).

a) b)

Figure 3.10: a) preparation of the mixtures for the biocrystallization experiment: final stirring, b) pipetted samples in dishes inside the inner chamber with two sensors for temperature and humidity monitoring. Cloudy mixtures: milk samples, clear-transparent samples: wheat standard.

Crystallization The dishes were placed onto the two rings in the chamber. 6 ml of the mixtures was pipetted in each of the dishes, see figure 3.10 b). The wheat extract was pipetted in a seven-fold repetition, each dish contained 90 mg wheat extract and 90 mg $CuCl_2$. Each of the six subsamples of milk (2x untreated, 2x treatment 1, 2x treatment 2) was pipetted in a six-fold repetition resulting in 200 mg of milk and 150 mg of $CuCl_2$ per dish. The pipetting order was randomized by a computer program (R, version 2.1.0). The crystallization process was monitored by a camera fixed to the ceiling of the inner chamber by taking a picture of the plates every 10 min. An example of the monitored crystallization process can be found in appendix C. After all samples were crystallized, the dishes were taken out of the rings and the inner chamber. The

acrylic rings were removed and the plates (crystallograms) were stored in the outer chamber until scanning. With the help of R and the photos, the nucleation time was determined. The average nucleation time usually ranges from 12-15 hours.

The experiments were repeated two days later[3] in a different randomized order. The crystallograms of both days were grouped together for statistical reasons.

Scanning The scanning of the pictures was performed with a UMAX PowerLook III transmitted light flatbed scanner and a target for colour correction. Four plates were scanned simultaneously at 600 dpi, 24 bit RGB colour without automatic colour correction and saved in the .tiff format to prevent information loss.

3.4.4 Picture evaluation

To obtain a highly objective opinion about the differences in the images caused by the different treatments and intensities, four different types of image analysis were applied. The images obtained with all three PFMs were evaluated via an online test and via a simple descriptive test conducted by the author. For the images obtained with biocrystallization, two other computer-assisted analyses were available: texture and structure analysis, respectively. Beforehand, unusable pictures were sorted out. For Steigbild and circular chromatographic pictures, these were pictures with inhomogeneous or generally poor flow behaviour. For the biocrystallization pictures, all pictures with a crystallization time longer that 15 hours were sorted out as those had specific characteristics that could be mistaken with differences caused by structural changes.

Online-picture-evaluation (all PFMs) Image analysis was based on a sensory test, namely the triangle test (DIN EN ISO 4120:2007) with modifications as discussed below. The basic task in a triangle test is to determine the deviating sample out of three samples that are handed out for evaluation simultaneously. The other two samples come from the same group and should therefore be recognized as equal or similar.

In this case, three images were evaluated by a test person simultaneously, two images obtained from the same sample group and one image of a different sample group. For example, two Steigbild images of untreated milk and one of heat treated milk at 85 °C for 5 min were shown for evaluation. The testers then had to tell which picture deviated from the others (test of distinction). Tests were performed with forced-choice, meaning an answer had to be given, even when the testers were uncertain about the correct answer.

For each of the four treatments and the three PFMs, nine different triad compositions were evaluated as shown in table 3.2. The nine triads were repeated once by

[3] one day was needed in between to stabilize the climate in the chamber.

swapping the sample groups for the double and single images relative to the first run making 18 triads per treatment per PFM. For the pictures obtained from the skimming experiments, only four different triad compositions (eight in repetition) were examined as only one treatment intensity existed. The same counts for the biocrystallization: only one intensity was examined for all the four treatments.

Table 3.2: Different questions (triad compositions) for each treatment and picture forming method in the online-picture-evaluation

question no.	treatment	experiment	vs.	treatment	experiment
1	UT[1]	1	vs.	T1[2]	1
2	UT	1	vs.	T2[3]	1
3	T1	1	vs.	T2	1
4	UT	2	vs.	T1	2
5	UT	2	vs.	T2	2
6	T1	2	vs.	T2	2
7	UT	1	vs.	UT	2
8	T1	1	vs.	T1	2
9	T2	1	vs.	T2	2

[1]untreated

[2]treated with low intensity

[3]treated with high intensity

As the amount of triads was very high (156 triads in total for all PFMs and treatments), the choice was made to perform the picture evaluation online, via a website. The website was created by *holosphere r&d* (Wageningen, the Netherlands). Students from the Institute for Food Technology and Food Chemistry among others were invited to join the evaluation via e-mail. Three tests were constructed: one for each of the three PFMs containing all the triads of all the treatments. In total, the tests for Steigbild and circular chromatography consisted of 62 triads and the test for biocrystallization of 32. The testers were sent a link for a test which was randomly chosen for them. They were allowed to also perform the other two tests. It was made sure that the testers did not repeat the tests. Some screen-shots of the website are shown in appendix D.

For each test, an introduction of the methodology was given. Then the testers were shown the triads, one at a time. The three images were chosen randomly from the pictures of the sample groups according to the criteria of table 3.2 and were shown aligned in a row. The sequence and composition of the 62 and 32 triads, respectively, were randomized for each tester. The testers had to click on the image they thought was the one deviating one out of the three. A red frame appeared around the chosen image and the testers were asked to press a 'Next' button to evaluate a new triad. If the testers wanted to change their minds before pressing next, they could press a 'Retry' button and were shown a triad of the same test question again, however

with new randomly chosen images. New triads were shown until all the triads had been evaluated. If the testers chose not to finish the test, some problems with the connection or the server occurred, the answers given until that point were counted as valid anyways.

The tests could be performed within three weeks. The collected data from all testers data was stored in a database for later evaluation. The statistical evaluation was performed with the help of table A1 of DIN EN ISO 4120:2007.

Simple descriptive test (all PFMs) To characterize the morphological features of the chromatograms and crystallograms, a simple descriptive test was carried out by the author. Hereby, all chromatograms and crystallograms made from each single sample were taken into account and the main common characteristics were noted down. As no descriptive test was performed by other test-persons, the results might be biased. However, it was beyond the scope of this thesis to also perform a descriptive test with a panel according to sensory norms. The results of this simple descriptive test will be used to understand the results of the online triangle test as it is important to know, on what characteristics the test-persons might based their choices.

Link between protein concentration and properties of the circular chromatogram As mentioned above in section 2.4, Knorr (1982) proposed a link between protein concentration and 'area of lowest optical density' in the circular chromatogram, meaning the whitish area around the center. On the basis on this paper, radii of the areas were determined with a ruler averaging two readings per chromatogram at an angle of 90°. For each realized experiment, 8-12 chromatograms were probed and the mean area was calculated. Subsequently, the results were linked with the protein concentrations determined by the Bradford method.

Texture and structure analysis (biocrystallization) For the evaluation of the biocrystallograms, two other computer based analyses were used: texture analysis and structure analysis (for the latter see below). For both, all plates per sample of the repeated experiments were grouped together.

Texture in this sense is not meant in a food related sense like the thick texture of a creamy yoghurt, but rather as "a region in 2D or 3D that can be perceived as being spatially homogeneous in some sense" (Carstensen, 1992, p. 53). Therefore, the term texture analysis also has a different meaning. The texture analysis used here was developed by Carstensen (1992); Andersen et al. (1999, 2001). Different ROIs, circular areas around the geometrical center of the glass plate were considered, compare figure 3.11, starting from 20 % up to 90 % of the total area in steps of 10 %. Basically, texture analysis compares and evaluates the distribution of differences in greylevel for neighbouring image points over a certain ROI and thus "reflects the spacial linear

relationships between grey-scale values" (Doesburg and Nierop, 2013, p. 63).

a) b) c)

Figure 3.11: a) ROI of 50 % (non blue area), b) ROI = 80 % and c) ROI = 100 %.

15 second order variables[4] were computed with ACIA 1.2.3 (Applied Crystallogram Image Analysis) software using the the Greylevel Co-occurrence Matrix (GLCM) algorithm: Energy, Entropy, Maximum probability, Correlation, Diagonal moment, Kappa, Difference energy, Difference entropy, Inertia, Inverse difference moment, Sum energy, Sum entropy, Sum variance, Cluster shade und Cluster prominence (see Kahl, 2007; Doesburg and Nierop, 2013).

As a second and relatively new Matlab (version 7.7.0 R2008b, Mathworks Inc., Natick, USA) based analytical tool, structure analysis was used, based on Doesburg and Nierop (2013).

The scanned RGB-images (crystallograms) were converted into greyscale. From this, a binary crystallization structure (BCS) was computed. For details about this transformation, see Doesburg and Nierop, 2013, p. 64. This BCS can be characterized by 15 parameters of each length and width of the crystal branches or needles.

The 16 parameters of width represent local maxima of different logarithmically evenly distributed diameters ranging from up to 2 pixels (D20) to broader than 13.4 pixels (D150). D24, for example, represents a diameter between 2 and 2.4 pixels, D29 , For that, dD73, D83, D94, D107 and D120. The 16th parameter D0 represents the all widths combined. The results were illustrated using different colours for each of the 16 parameters. So, each local maximum represented a differently coloured droplet resulting in a Van Gogh like 'painting', compare figure 3.12. Quantification of the results was obtained by measurement of the covered surface at different ROIs, ranging from 20-90 % at 10 % steps.

For the evaluation of the needle lengths, 19 parameters were used: needle starting points, also called nodes (parameter: Lnode) and end points (Lend) were differentiated. Special procedures were applied for very short (L0) and specially curved or inter-winded branches (Lsplit). Furthermore, a similar approach to the evaluation of the needle widths was used. The 15 remaining parameters of length represented local maximum

[4]The names of these variables do not have anything to do with physical variables.

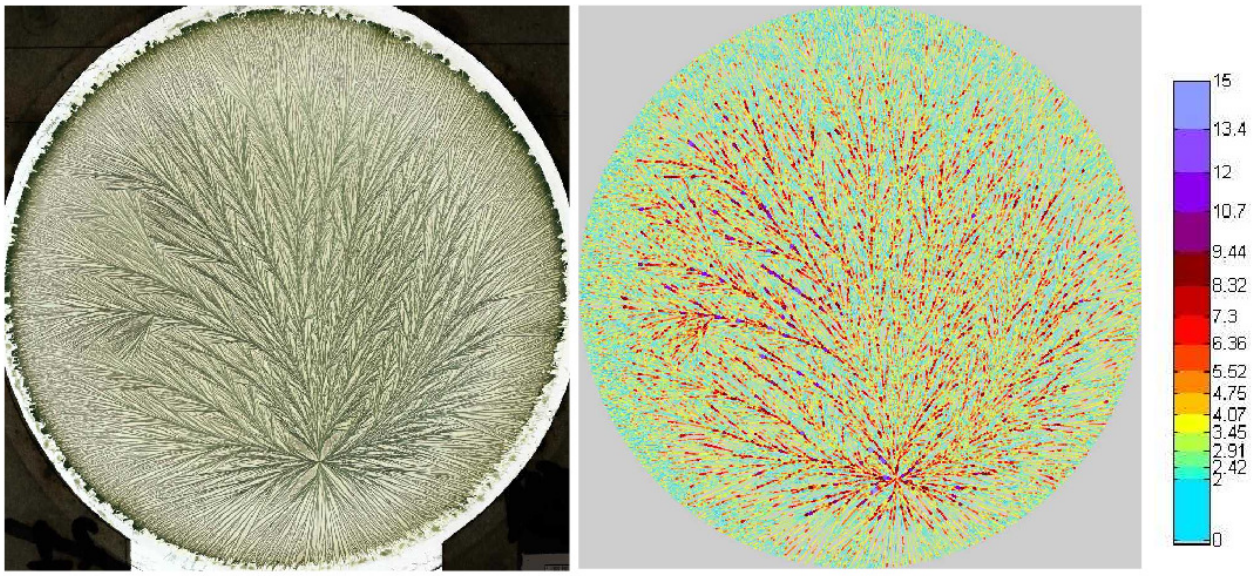


Figure 3.12: Original scanned RGB image untreated milk (left) and the structure analysis characterization of the category width. Pixel diameter-ranges are represented by different colours.

of different logarithmically evenly distributed lengths ranging from up to 2 pixels (L20) to longer than 22 pixels (L250). L26 represents a needle length between 2 and 2.6 pixels. This scheme likewise counts for the residual parameters L33, L41, L51, L62, L75, L89, L108, L124, L144, L167, L192, and L220. The results were illustrated using different colours for each of the 15 parameters. So, each local maximum represented a differently coloured line of a width of one pixel. Nodes, split and end points were marked as single pixels, coloured grey, wight and black, respectively. The outcome looked like a line drawing. Quantification of the results was obtained by measurement of the covered surface at different ROIs, ranging from 20-90 % at 10 % steps. For more details, see Doesburg and Nierop, 2013, pp. 64f.

The data of both, structure and texture analysis was statistically evaluated with aid of the program R (version 2.1.0), using a 'linear-mixed-effects' (LME) model by means of crossed effects with repeated measurements. Mixed effects hereby means that both, random and fixed effects are incorporated in the model. After Meelursarn (2007, p. 15) "[t]he fixed effect is the effect which parameters are associated with an entire population or with certain repeatable levels of experimental factors. While, random effect is the effect which is associated with individual experimental units drawn at random from a population."

R-cran was used to perform box-plots and Principal Component Analysis (PCA). These were plotted against the ROIs (20-90 %). Previous to further data analysis, outliers were being identified and excluded if they were bigger than the value of four times the standard deviation of the variable for a specific ROI.

The outcomes were presented in the following graphs:

- PCA score-plot over ROI. PCA is used to reduce and interpret the large multivariant data sets and to discover previously unsuspected relationships. PCA allows to use all parameters (for example for width) simultaneously and compose so called component vectors. The two best describing components are plotted. The circles represent groups of data points that are similar. One can say that the less the groups of data points overlap in the score-plot, the higher the likelihood is that the data sets differ significantly. In our case, the less the groups of data points overlap, the bigger the effect of the treatment on the crystallization pattern. For more details about PCA see for example Jolliffe (2002).

- Box-plots of log(p) over ROI (LME-ANOVA) for the effects of treatment, experimental day and their interaction. Log(p)-values were hereby calculated for the difference between the crystallization patterns between untreated and treated samples. A p-value < 0.05 was considered significant. For more information see Meelursarn (2007).

- Box-plots showing the number of crystals of a certain width/length over ROI to show whether the treatment had an effect on the size of the crystals.

- Box-plots showing the interaction between the crystallization starting time (nucleation time) and the combined length-variables, represented by the $\bar{R}^2$ (adjusted R^2 - adjusted coefficient of determination) over ROI. $\bar{R}^2$ was used as R^2 has the tendency to deliver falsely higher values when additional explanatory variables are added. $\bar{R}^2$ corrects this and is thus always below or equal R^2. The value of $\bar{R}^2$ usually lies between 0 and 1, however, also negative values are possible. A result of $\bar{R}^2 = 0.8$ can be interpreted as 80 % of the observed variance is due to a linear relationship with one or several factors, here: evaporation time. For more information see for example Ireland (2010); Field et al. (2012).

4 Results and discussion

In this chapter, the results obtained with standard analytical (HPLC, protein and fat determination) as well as with picture forming methods (Steigbild, circular chromatography and biocrystallization) are discussed and compared. It is outlined to which degree the PFMs are able to detect protein denaturation in raw bovine milk caused by different treatments and whether they are even more sensitive and able to identify other changes that are not detected with the standard analytical methods.

4.1 Results obtained with standard analytical methods

In the following the outcomes of the HPLC-measurements as well as the result of the determinations of protein and fat contents are presented and compared to findings in literature.

4.1.1 HPLC-results

In the following, the changes in protein structures are discussed as they were detected with HPLC. The graphs each represent the average of all conducted experiments. The results are highly reproducible as one can see by the relatively small standard deviations, compare figures 4.1, 4.2 and 4.3. However, as separation of the whey proteins was not precise enough for some samples, the results for the single whey proteins (β-lactoglobulin A & B, α-lactalbumin) were grouped together. The weak separation is probably partially caused by fluctuations of the column temperature as well as sample specific characteristics which are unknown. Results of the casein fractions (α-casein, β-casein and κ-casein) were grouped as well, since the single fractions showed very similar behaviour.

As one can see in figures 4.1, 4.2 and 4.3, the whey protein contents of the centrifuged samples were in most cases higher than the contents of the untreated samples, in some cases up to 12 %. A possible explanation might be a residual fat content in the untreated samples as those samples were not centrifuged and thus fat only was separated via filtration (compare section 3.3.1). This would lower the relative content of whey proteins. As the differences caused by the treatments are more pronounced than those caused by this phenomenon, the results remain meaningful.

Another recurring phenomenon is the lower whey protein content of the untreated samples at pH 4.6. Apparently, some of the whey proteins are lost during centrifugation. This suggests that a part of the whey proteins in the untreated samples interact

with the caseins or is denatured. As mentioned before in section 2.1.1, β-lactoglobulin forms octamers between pH 3.5 and 5.5, and specially at pH 4.6. Very likely, those bigger octamers were separated by centrifugation and were lost for further analysis.

HPLC-results of the heat treated samples Figure 4.1 shows the HPLC-results of the heat treated samples. From this follows that even the intense heat treatment (85 °C for 5 min) did not show any detectable effect on the caseins. No change in size was detected as the casein content of the serum phase did not differ with HP treatment. This is an expected result, as caseins are highly heat stable (compare section 2.2 and Töpel (2004)). If casein cell size had increased or decreased, less or more casein would have been found in the serum phase, respectively. Also, no aggregation of caseins with whey proteins (above all denatured β-lactoglobulin) took place as to changes of contents in the serum phase were observed. It has to be mentioned that possibly undetected denaturation could have taken place in the insoluble fraction of the caseins that was separated by centrifugation during sample preparation. Furthermore, no increase was detected for the samples at pH 4.6 which shows that HP does not seem to have an influence on the structure of the (sub-)micelles.

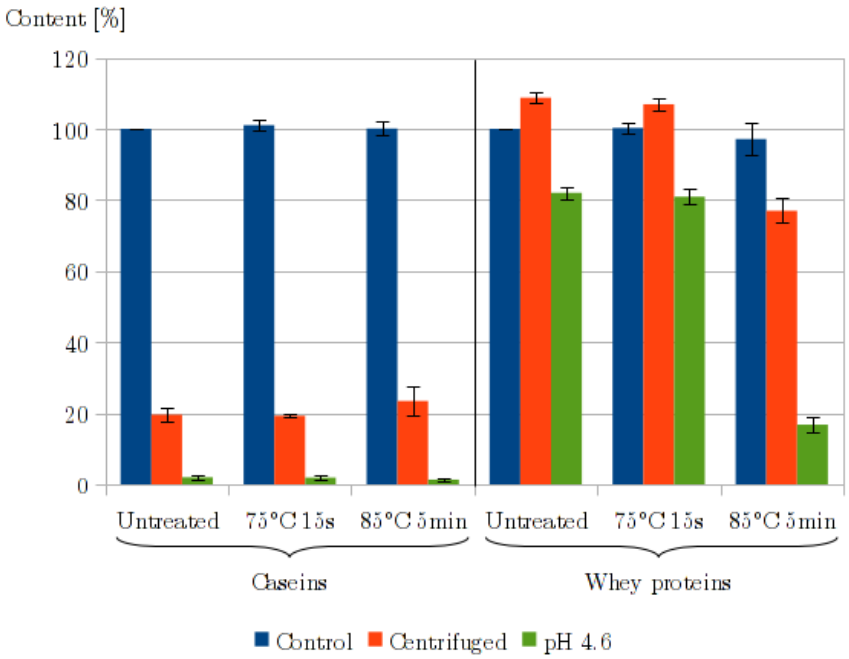


Figure 4.1: HPLC-results of the heat treated samples.

However, the case is different with the whey proteins, see figure 4.1. The more intense treatment probably led to the denaturation of β-lactoglobulin and most likely also α-lactalbumin (see also figure 2.8 on page 12). More than 80 % of the whey proteins are lost from the soluble fraction at pH 4.6 85 °C. The decreased amount of whey proteins in the serum phase (red bars) give the hint that, at 85 °C, the whey proteins interacted with the casein micelles and thus were able to be removed via

centrifugation. Most likely, aggregation of β-lactoglobulin took place as denaturation led to the uncovering of free thiol-groups in the β-lactoglobulin molecules (Considine et al., 2007). Probably this led to a decreased denaturation of α-lactalbumin as less free thiol-groups were available with the aggregated β-lactoglobulin molecules. As discussed in section 2.2, α-lactalbumin can only denature and bind to other molecules when free thiol-groups are available. However, due to poor separation the exact behaviour of α-lactalbumin and and β-lactoglobulin A & B is left unclear.

With the samples at pH 4.6 one can see the interactions of caseins and whey proteins even more clearly. As the caseins fell out, the whey proteins attached to the casein surface probably precipitated together at pH 4.6. It is possible that the whey proteins also partially interacted with the proteins of the fat globule membrane at 85 °C, compare section 2.2. Separation then could have happened during centrifugation and filtration.

At pasteurization conditions (75 °C for 15 s), no significant effects on whey proteins were observed. After Töpel (2004), β-lactoglobulin denatures significantly and irreversibly at temperatures around 70-75 °C. Very likely, the treatment time was too short to induce significant changes. However, Töpel (2004) does not report after which treatment time the losses could be detected.

In summary, intense heat treatment of milk was found to lead to whey protein denaturation and subsequent interaction with the casein micelle surface.

HPLC-results of the HP treated samples The outcomes shown in figure 4.2 paint a different picture: After centrifugation of the HP treated samples, more caseins remain in the serum phase. This means, the average size of the casein micelles decreased as the casein micelles were disrupted into smaller sub-micelles which is in conformation with literature (Hinrichs, 2000; Huppertz et al., 2004d, 2006; Considine et al., 2007). The higher the pressure, the stronger is the micellar disruption and therefore the amount of casein sub-micelles, which was separated via centrifugation less easily, increases. In literature the disintegration of the casein micelles is being explained by the weakening of ionic bonds and hydrophobic interactions (Hinrichs, 2000). Large aggregation of caseins and whey proteins was not observed as otherwise, the content of caseins in the serum phase would have decreased. Furthermore, no increase was detected for the samples at pH 4.6 which shows that HP does not seem to have an influence on the structure of the (sub-)micelles.

As discussed earlier in section 2.2, it is possible that the casein micelles for the samples treated at 300 re-associate again (Huppertz et al., 2004d). This phenomenon, however, was not examined here. Compared to the untreated milk samples, the HP treated ones were slightly more translucent which also points to the disruption of the casein micelles.

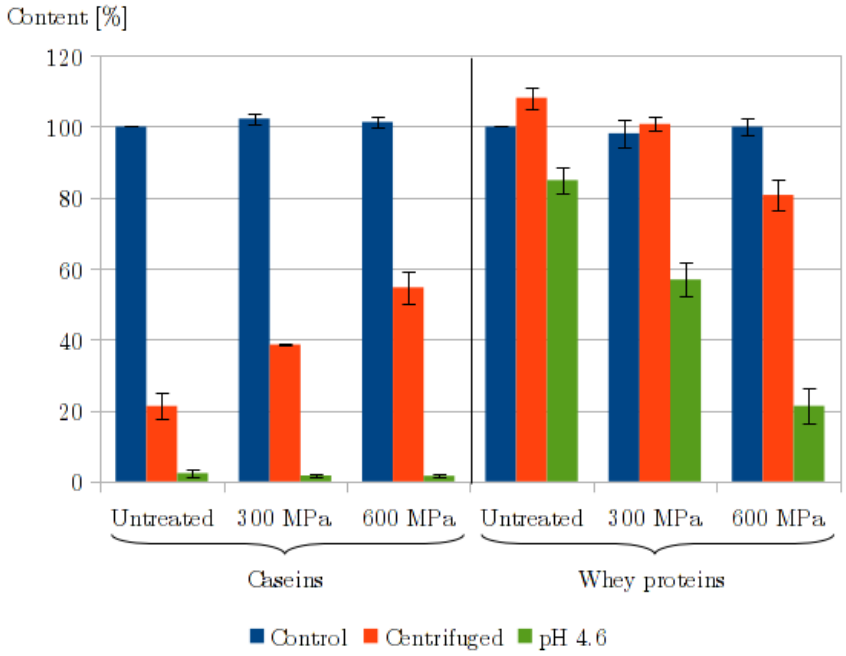


Figure 4.2: HPLC-results of the HP treated samples.

The decreasing whey protein content in the serum phase hints towards the binding of denatured whey protein molecules to the casein surface for the HP treated samples. Most likely, aggregation of β-lactoglobulin took place which then again might have led to a decreased denaturation of α-lactalbumin (see discussion above). The bound whey protein molecules and the aggregates then were separated by centrifugation together with the casein molecules. Whereas this effect was rather weak at 300 MPa, about 20 % of the whey proteins were no longer dissolved at 600 MPa which is in line with literature (Gaucheron et al., 1997; Hinrichs, 2000; Hinrichs and Rademacher, 2005; Huppertz et al., 2006; López-Fandiño, 2006; Considine et al., 2007).

Once again, whey protein denaturation was visible more clearly with the samples at pH 4.6. Slightly less than half (43 %) of the initial whey protein content in the serum phase was lost at 300 MPa. At 600 MPa, another third bound to the caseins and was precipitated. Probably, the remaining serum proteins consisted mostly of α-lactalbumin as it is more pressure-resistant. At 300 MPa, mainly the β-lactoglobulin molecules must have lost their native state as the denaturation of α-lactalbumin only starts above 400 MPa (Huppertz et al., 2004b, 2006).

To conclude, it was observed that HP treatment of milk leads to the disruption of casein micelles into sub-micelles and to the denaturation of whey proteins and their interaction with the casein surface.

HPLC-results of the PEF treated samples As one can see clearly in figure 4.3, no significant structural changes occurred with caseins and whey proteins. Even a

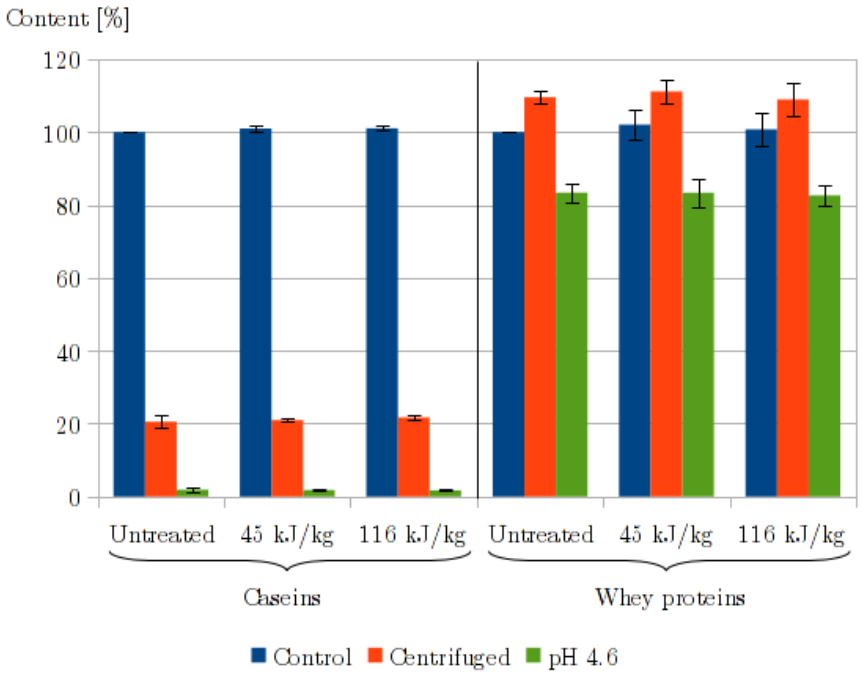


Figure 4.3: HPLC-results of the PEF treated samples.

high energy input of 116 kJ/kg did not lead to any significant protein denaturation or aggregation. These observations are in line with the results of other authors such as Barbosa-Cánovas et al. (1998); Barsotti et al. (2001); Michalac et al. (2003); Perez and Pilosof (2004); Odriozola-Serrano et al. (2006). The observed disruption of casein micelles by Floury et al. (2005) only happened at high maximum field strengths between 45-55 kV/cm. In these experiments, the maximum field strength of 10 kV/cm was probably too low to show any effect.

4.1.2 Protein and fat contents

As milk is a natural product, its composition changes over time due to several factors such as the lactation period of the cow (see section 2.1). Figure 4.4 shows the protein and fat contents of the untreated samples sorted by experiment. It illustrates the fluctuations of protein and fat content among the experiments of the single treatments. As one can see clearly, protein and fat contents were not stable but underwent natural fluctuations: protein content ranged between 3.03 and 3.80 %, whereas the fat content fluctuated between 3.54 and 4.70 %. The greatest fluctuations were observed between two experiments of heat treatments H-1 and H-2. Here, the difference was 1.12 % for fat content and 0.57 % for protein content, respectively. Fluctuations over time are shown in appendix E.

Figure 4.4 also illustrates the measured fat contents of the skimmed samples (Z-1 and Z-2). Among the four experiments, fat contents ranged between 0.02 and 0.11 %, suggesting that the size distribution of the milk fat globules also changed over time

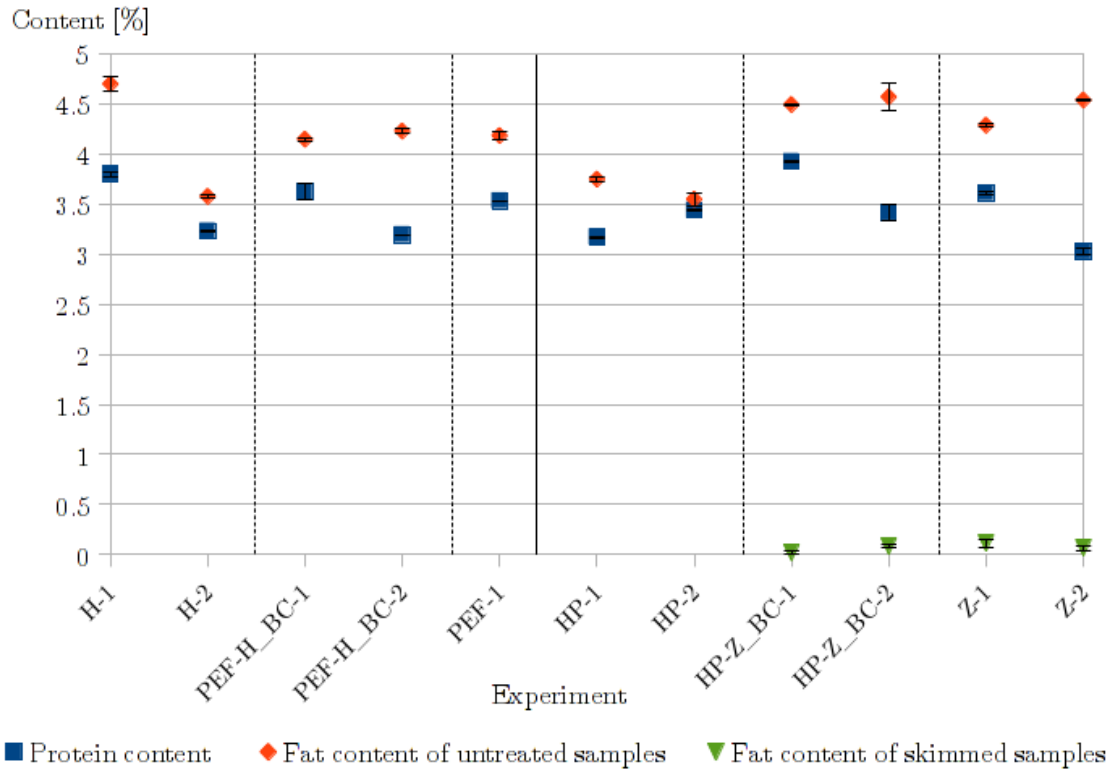


Figure 4.4: Protein and fat contents of untreated samples and fat contents of skimmed samples, sorted by experiment. The experiments labeled with BC represent the biocrystallization experiments whereby always two treatments were applied with only the higher intensity each. Experiments not labeled BC represent the experiments conducted with Steigbild method and circular chromatography, which were evaluated together. H: heat treatment, Z: skimming.

which is in accordance with literature (Töpel, 2004, p. 388).

With the help of the HPLC-results and obtained total protein contents, concentrations of the different protein fractions were determined. Figure 4.5 shows the average concentrations of total proteins, caseins and whey proteins of all raw milk samples. On average, the protein content was 34.9 g/l, whereby the concentration of caseins was 30.2 g/l and the whey proteins took the remaining part of 4.7 g/l. Of all proteins, the casein fraction was 86.5 % which is rather high, compare Töpel (2004); Baltes (2007). However, as not all whey proteins were detected by HPLC, the results do not reflect the actual fragmentation. The fraction of whey proteins would have been higher if the remaining whey proteins (bovine serum albumin, immunoglobulin, proteose-peptone) had been taken into account. The standard deviation shows that the total fluctuations were higher for caseins than for whey proteins.

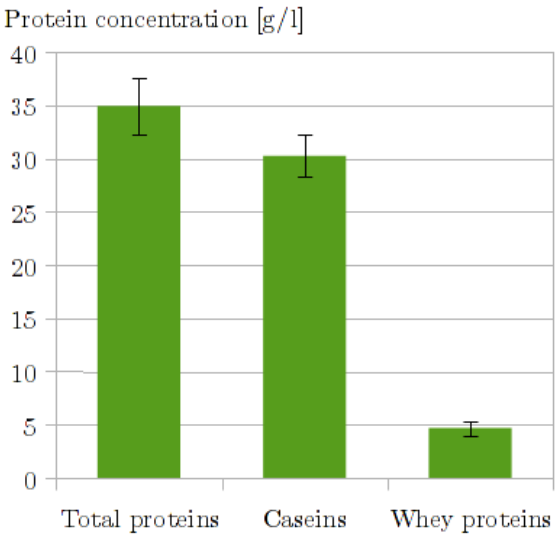


Figure 4.5: Average concentrations of total proteins, caseins and whey proteins of all milk samples.

4.2 Results obtained with picture forming methods

In the following, the results of the online visual evaluation of the chromatograms and crystallograms are discussed. To also include descriptions and comparisons of morphological characteristics, a simple descriptive visual evaluation was undertaken.

85 persons participated in the online tests, of which twelve took part in more than one of the three tests for Steigbild, circular chromatography and biocrystallization, respectively. The number of participants differed slightly among the tests. It was observed that several people did not finish the test, in total 21 %. One can conclude that the test design with 62 and 32 triads, respectively, was experienced as too long. Early drop-out maybe could have been prevented if for example information about progress had been given.

In the following, only results with a p-value ≤ 0.05 will be considered significant. Furthermore, the term 'sample' now refers to the repetitions of the experiments with different milk sampling on different days and is not to be confused with the (un)treated milk (samples). As mentioned in section 3.4.4, the number of triads shown to the test subjects was doubled by swapping the sample groups for the double and single images. Thus, the number of answers given is always higher than the number of test subjects.

4.2.1 Steigbild results

The online image analysis test of the Steigbild pictures was performed by 32 persons from which 15 % ended the test prematurely. The results of the online image analysis test showed a lot of highly significant differences, see table 4.1. By observing morphological criteria, differences were mostly observed in the bowl zone and the flag zone.

Only those morphologic characteristics that differ among the pictures are mentioned below. Example images of all treatments and intensities are shown in figure 4.6.

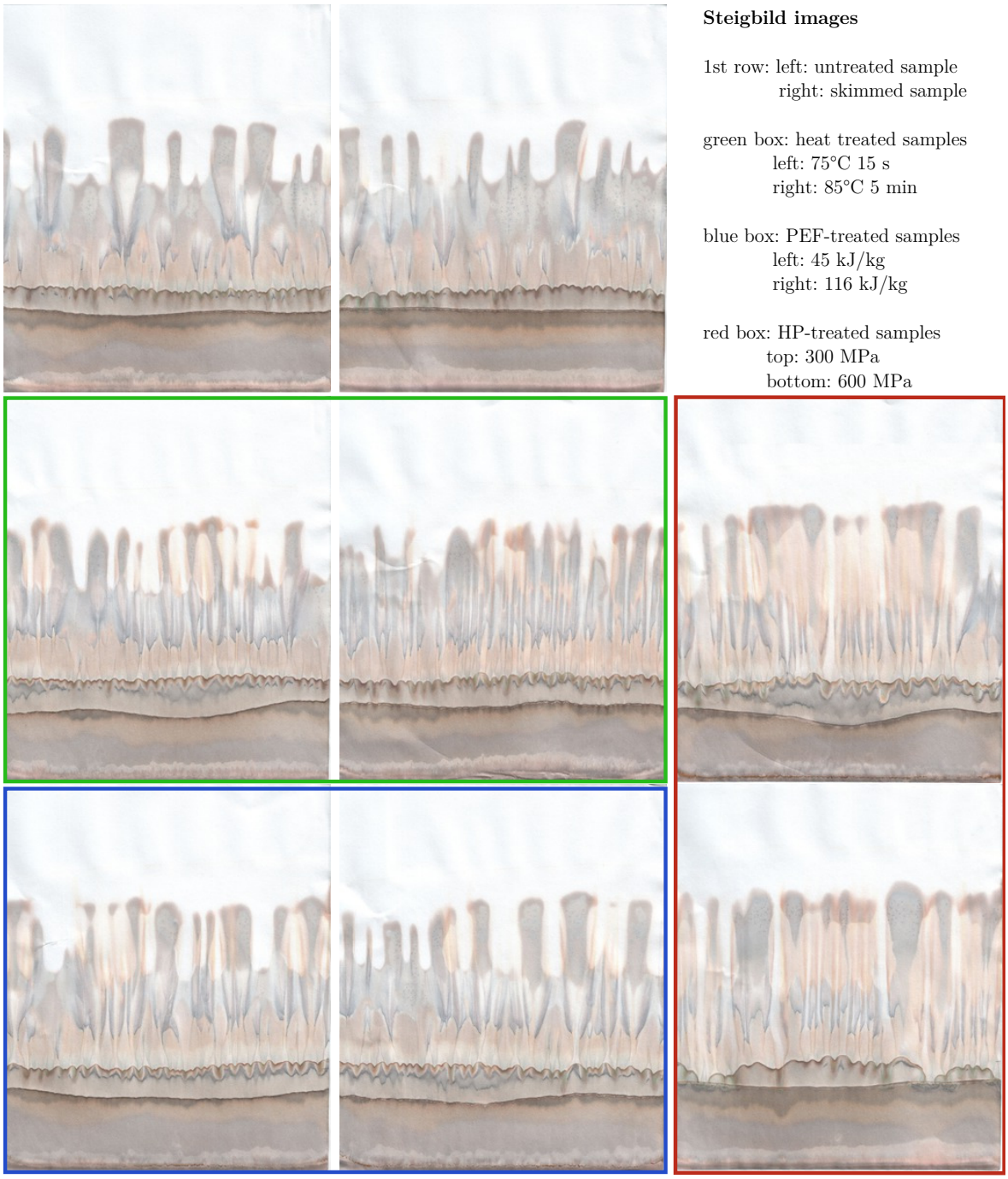


Figure 4.6: Selected Steigbild images of the various treatments. Images are chosen from both sample repetitions.

Results of the HP treatment When looking at the morphology, chromatograms of untreated milk usually showed the following characteristics: few and rather broad flags that were sometimes poorly defined and seemed to 'flow' into each other. The bowls

Table 4.1: Overview of the results of the online image analysis for the experiments conducted with the Steigbild method. Xi: no. of repeated experiment (sample), 000: untreated milk, 300 / 600: MPa, treatment intensity for HP treated milk, 075 / 085: °C treatment intensity for heat treated milk, 045 / 116: kJ/kg, treatment intensity for PEF treated milk, 100: skimmed milk. - : no significant differences found: $p > 0.05$

treatment	**question * vs. ***		**total answers**	**correct answers**	**% correct answers**	**p-value**
	X1/000	X1/300	62	31	50.00	< 0.01
	X1/000	X1/600	61	27	44.26	0.05
	X1/300	X1/600	61	19	31.15	-
	X2/000	X2/300	60	45	75.00	< 0.001
	X2/000	X2/600	59	35	59.32	< 0.001
	X2/300	X2/600	59	37	62.71	< 0.001
HP	000	300	122	86	70.49	< 0.001
	000	600	120	62	51.67	< 0.001
	300	600	120	56	46.67	< 0.001
	X1/000	X2/000	59	33	55.93	< 0.001
	X1/300	X2/300	59	51	86.44	< 0.001
	X1/600	X2/600	59	37	62.71	< 0.001
	X1/000	X1/075	58	18	31.03	-
	X1/000	X1/085	58	39	67.24	< 0.001
	X1/075	X1/085	58	40	68.97	< 0.001
	X2/000	X2/075	58	15	25.86	-
	X2/000	X2/085	58	46	79.31	< 0.001
	X2/075	X2/085	58	51	87.93	< 0.001
HEAT	000	075	116	33	28.45	-
	000	085	116	85	73.28	< 0.001
	075	085	116	91	78.45	< 0.001
	X1/000	X2/000	58	26	44.83	0.05
	X1/075	X2/075	58	23	39.66	-
	X1/085	X2/085	58	26	44.83	0.05
	X1/000	X1/045	57	18	31.58	-
	X1/000	X1/116	57	10	17.54	-
	X1/045	X1/116	57	16	28.07	-
	X2/000	X2/045	56	12	21.43	-
	X2/000	X2/116	56	31	55.36	< 0.001
	X2/045	X2/116	56	25	44.64	0.05
PEF	000	045	113	31	27.43	-
	000	116	113	41	36.28	-
	045	116	113	41	36.28	-
	X1/000	X2/000	56	26	46.43	< 0.05
	X1/045	X2/045	55	23	41.82	-
	X1/116	X2/116	55	39	70.91	< 0.001
	X1/000	X1/100	54	27	50.00	0.01
	X2/000	X2/100	54	23	42.59	-
SKIM	000	100	108	50	46.30	< 0.01
	X1/000	X2/000	54	31	57.41	< 0.001
	X1/100	X2/100	54	23	42.59	-

were small and showed a clear corona (whitish edge), few reduction spots were visible in the upper part of the flags (compare figure 4.6, first row, left image). In the Steigbild images of the milk samples treated with 300 MPa for 10 min some characteristics differed: flags were usually less broad and their amount was higher. This finding, however, could not be confirmed by literature: Balzer-Graf and Gallmann (2000) found the flags to be more broad and less defined. The report however does not specify the exact methodology and thus their results have to be handled with care. Bowls were slightly bigger and broader than for the untreated samples. The Steigbild images of the milk treated at 600 MPa for 10 min showed similar characteristics in the flags. Generally, their number was higher and they were thinner, even compared to the samples obtained from milk treated at 300 MPa. The occurrence of the formation of bigger bowls for the HP treated samples might be explained by differences in the solubility compared to the untreated samples (compare section 2.4.1).

As discussed in section 2.4.1, the flags of Steigbild images are formed by means of local flow delay, caused by the substances in the sample and the resulting formation of a boundary lines between two adjacent zones of flow, which are marked with reduced silver atoms of brownish colour. The interactions of casein (sub-)micelles and whey proteins probably led to increased blocking of the filter pores, which caused the appearance of a higher number of flags for the chromatograms of the HP treated samples. As the interactions between whey proteins and caseins were found to be greater for the samples treated with 600 MPa, the higher number of flags present in the Steigbild images can be explained. It is interesting to note that no large aggregates were formed during and after HP treatment as the results shown in figure 4.2 suggest. Large aggregates usually lead to blocking of the filter pores and could therefore explain the occurrence of rising inhibitions. It might be possible that, in contact with the salt solutions, aggregates were formed.

For the samples treated at 600 MPa, often, a rising inhibition occurred in the Steigbild pictures in the second rising phase ($AgNO_3$), which is not the case for the untreated milk and the samples treated at 300 MPa, compare figure 4.6, red box. The latter was also observed by Balzer-Graf and Gallmann (2000) who found rising inhibitions for milk samples treated with 500 and 700 MPa, respectively, for five minutes. It can be theorized that, again, the interactions of whey proteins and caseins formed during HP treatment led to (partial) blocking of the filter pores which then hindered the $AgNO_3$ from rising further. As the interactions between whey proteins and caseins were higher for the samples treated at 600 MPa, compare figure 4.2 on page 67, probably the blocking of the filter pores was increased and occurred already in an earlier rising phase.

Looking at the results shown in table 4.1, one can say that the testers were able to differentiate the Steigbild pictures obtained with the HP treated milk very well from the ones of untreated milk. This counts especially for the second sample, where

differences were highly significant ($p < 0{,}001$). The variance in the pictures of the first sample between the untreated and treated samples was slightly less clear. Furthermore, here milk treated at different intensities did not show significant differences. When summarizing the result of the two sample repetitions (000 vs. 300, etc.), the overall outcome still was highly significant ($p < 0{,}001$).

However, as the previously discussed results already indicated: the Steigbild pictures of the two different samples (X1/000 vs. X2/000, etc.) show different morphological features and variations are highly significant ($p < 0{,}001$ for all). This is probably due to the occurrence of reduction spots as they only accumulated in the pictures of the second sample, compare figure 4.6, red box. Also, an extra greyish layer appeared under the bowls of the Steigbild pictures of the second sample which made the pictures easy to differentiate.

The differences in fat and protein content of the two milk samples are only small (0.2 and 0.27 %, respectively) and thus it is unlikely that these variations in composition caused such big morphological modification. Either outer or inner fluctuations might have caused the effect, for example temperature and humidity in the outer room where the climate cabinet was standing were not controlled. Thus the climate at the start of the experiments might have differed. This might have had an effect on the rising behaviour of the solutions and the inherent compounds. Also, as Wohlers (2011) already suggested, the climate also has an influence on the chromatographic paper. Thus it should have been stored in a climate controlled room as well. Furthermore, the temperature of the samples at the beginning of the experiments might have differed. Lower temperatures lead to an increase in viscosity and therefore a slower rising of the solutions. Additionally, the age of the salt solutions might have played a role. The solutions were maximum one week old when used and thus lie in the two weeks range proposed in literature (Wohlers, 2011). Also, other undetected fluctuations might explain the findings. To this point, however, the exact cause remains unclear. Further experiments are needed to determine all the factors involved.

One can conclude that the Steigbild method is capable of showing differences induced by HP treatment of milk. Interactions between casein sub-micelles and whey proteins lead to rising inhibitions in the bowl zone and an increased number of flags.

Results of the heat treatment Concerning the heat treatment, highly significant differences were found between the pictures of the untreated milk and the ones treated at 85 °C for 5 min ($p < 0{,}001$). Also, pictures of milk treated at 75 °C were easily distinguishable from the ones treated at the higher intensity ($p < 0{,}001$). However, the testers were unable to differentiate the chromatograms of the untreated from the ones treated at 75 °C for 15 s. These findings count for both samples. The results are in line with the findings of the HPLC-analysis. No detectable denaturation or

interaction of the caseins and whey proteins was observed for the mildly heat treated samples, whereas significant whey protein denaturation and interaction with caseins was detected with the intense heat treatment.

Like with the HP treated milk, pictures of same treatments but different sample were distinguished by the testers, however less significantly. It can be guessed that the differences detected by the testers were due to variations in the protein and fat content as they differed strongly among the two samples: the difference in protein content was 0.57 % and in fat content 1.12 %. As discussed before, additionally unknown outer and inner conditions might explain the differences observed.

When looking at the morphology, it was observed that (intense) heat treatment had an effect on the flags in the Steigbilder. Both mild and intense heat treatment led to an increased number of flags, compare figure 4.6, green box. Additionally, the flags were less broad but ordered in a parallel manner. A higher treatment intensity led to more and thinner flags. The number of flags for the intense treatment was even higher than for the HP treated samples. No clear differences could be observed in bowl and base zone.

When we compare this to the findings above of the HP treated milk, we could assume that the denaturation of the whey proteins and their interactions with the caseins might have led to the alteration in the 'gestalt' of the flags, as thinner and more rigid flags were also observed with HP treated milk, where whey protein denaturation and interaction with caseins happened as well. Unlike HP treatment, casein micelles were not disrupted during heat treatment. This means, the bigger casein micelles covered with denatured whey proteins are more likely to block the filter paper due to their bigger size, compared to the HP treated samples, where caseins mainly exist in the form of smaller sub-micelles. Also here, aggregates could have formed in the contact with salt solutions.

Wohlers (2011) studied how heating of milk at 100 °C influences the morphological characteristics of the Steigbilder. She also found the flags to be thinner and more parallel. Additionally, she observed that the flags were more 'rigid' and the 'flag legs' were less intensive. Next to that she encountered differences in the bowl zone: It looked 'washed-out' and were less differentiated (p. 177). It can be guessed that the formation of new compounds, for example during the Maillard reaction that happens more rapidly at those temperatures, might have led to the differences observed in the bowl zone.

To conclude, the Steigbild results were in line with the ones obtained by HPLC. Differences were detected between the untreated samples and the ones of the intense heat treatment. These variations probably reflect the denatured whey proteins and their interactions with the caseins.

Results of the PEF treatment Concerning the images obtained by the PEF treated samples, no clear significant differences between the images of the untreated and the treated milk samples were detectable in general. However, two exceptions occurred: the testers identified significant variations between the images of the milk treated with 116 kJ/kg (high intensity) and the untreated ones ($p < 0{,}001$), but only for the second sample. Also, images of milk treated at 45 kJ and 116 kJ/kg of the second sample could be distinguished with the online image analysis ($p = 0{,}05$). These significant differences were not found for the first sample. How it came to these results remains unclear as no clear morphological differences were observed in the chromatograms of the PEF treated samples. Differences found usually occurred between repetitions and not between treatment intensities, see also table 4.1. Like with the HP treated samples, a greyish zone occurred under the bowls of the chromatograms of the second experiment, compare figure 4.6, blue box. It was observed that here, the bowl zone was slightly broader as well, suggesting that the occurrence of the greyish area was correlated to the size of the bowl zone. Probably, the differences detected therefore merely are caused by imprecision, as the glasses might have been put over the chromatograms for too long during the second rising phase and thus $AgNO_3$ was able to rise above the 1 cm marking.

The results give the hint that with the Steigbild method it is not possible to distinguish between untreated and PEF treated milk samples as no clear and repeatable differences occurred between the images of the PEF treated and untreated milk. This suggests that the state of proteins probably plays a major role in the pattern formation of the Steigbild images as effects were mainly observed for heat- and HP treatment but not for PEF treatment, where the proteins kept their native state.

Results of skimming The online image analysis gave significant results for the differentiation between untreated and skimmed milk for the first ($p = 0{,}01$) but not for the second sample. The combination of the two samples still led to a significant distinction ($p < 0{,}01$).

When one compares the chromatograms of the skimmed and the untreated milk, small changes were visible in the bowl zone of the images of the skimmed samples: the corona, the whitish edge under the bowls was missing (see figure 4.6, first row, right image), suggesting that the corona is somehow linked with the milk fat. It might be that the corona consists of milk fat which showed slower flowing behaviour due to relatively large droplet size compared to other milk compontents. The milk fat might then have accumulated under the bowls, as the filter pores were already blocked with other components, such as proteins (see above).

Unlike heat and HP treatments, changes in the flag zone were not observed. However, also here, highly significant differences ($p < 0.001$) were found between different

sample repetitions. This result does not count for the skimmed milk, though. Obviously, the skimming effect was bigger than the effect the different samples had on the chromatographic pattern.

It remains unclear to what extend it is possible to detect modifications of the fat content with the Steigbild method as the significant results found in one sample where not reproducible with a second one. However, when comparing the chromatograms of both skimmed samples, they seemed similar. A possible cause for the observed outcomes might be the differences found between the two untreated samples which probably did resemble more the chromatograms of the skimmed rather than the untreated milk. Yet, fat contents of the second sample (4.54 %) was even higher than for the first one (4.29 %). Also, the fat content of the skimmed milk was found higher for the first sample (0.11 %) than for the second one (0.07 %), compare figure 4.4. Thus the difference in fat content between the skimmed and untreated milk was higher for the second sample. Maybe, the difference in protein content (0.58 %) plays a role. However, it is also likely that differences in climate are linked with the occurrence of different patterns. The influencing factors can only be guessed.

To summarize, the effect of skimming on milk was detected with the Steigbild method. The milk fat is probably directly linked with the occurrence of the corona, the whitish edge below the bowls.

4.2.2 Results of circular chromatography

The online image analysis test of the circular chromatograms was performed by 28 persons from which 25 % ended the test early. Compared to the Steigbild method, fewer significant differences were found between the circular chromatograms of the untreated and the treated samples, compare table 4.2. The few alterations detected by the descriptive test are related to the differences in colour of the total chromatogram and the size of the rings in the inner zone. Only those characteristics that differ among the pictures are mentioned below. Example images of all treatments and intensities are shown in figure 4.7.

Results of the visual evaluation As the results of the online image analysis show in table 4.2, very few significant variances were found between the circular chromatograms of unprocessed and processed milk concerning all treatments. No differences were detected between untreated and treated milk in both experiments conducted with HP, PEF and skimming. Some significant differences were attained for the heat treated milk, with a higher probability for the low intensity ($p < 0.01$ and < 0.05, respectively). However, these findings could not be repeated with the second sample and thus the results lose their significance when combined. As no changes were detected

Table 4.2: Overview of the results of the online image analysis for the experiments conducted with the circular chromatographic method. Abbreviations see table 4.1 on page 72

treatment	question * vs. *		total answers	correct answers	% correct answers	p-value
	X1/000	X1/300	53	14	26.42	-
	X1/000	X1/600	52	11	21.15	-
	X1/300	X1/600	52	14	26.92	-
	X2/000	X2/300	52	16	30.77	-
	X2/000	X2/600	51	12	23.53	-
	X2/300	X2/600	51	9	17.65	-
HP	000	300	105	30	28.57	-
	000	600	103	23	22.33	-
	300	600	103	23	22.33	-
	X1/000	X2/000	51	49	96.08	< 0.001
	X1/300	X2/300	51	49	96.08	< 0.001
	X1/600	X2/600	51	40	78.43	< 0.001
	X1/000	X1/075	49	26	53.06	< 0.01
	X1/000	X1/085	49	23	46.94	< 0.05
	X1/075	X1/085	48	11	22.92	-
	X2/000	X2/075	48	12	25.00	-
	X2/000	X2/085	47	14	29.79	-
	X2/075	X2/085	47	16	34.04	-
HEAT	000	075	97	38	39.18	-
	000	085	96	37	38.54	-
	075	085	95	27	28.42	-
	X1/000	X2/000	47	42	89.36	< 0.001
	X1/075	X2/075	46	39	84.78	< 0.001
	X1/085	X2/085	46	41	89.13	< 0.001
	X1/000	X1/045	43	14	32.56	-
	X1/000	X1/116	43	12	27.91	-
	X1/045	X1/116	42	15	35.71	-
	X2/000	X2/045	42	16	38.10	-
	X2/000	X2/116	42	10	23.81	-
	X2/045	X2/116	42	15	35.71	-
PEF	000	045	85	24	28.24	-
	000	116	85	22	25.88	-
	045	116	84	30	35.71	-
	X1/000	X2/000	42	27	64.29	< 0.001
	X1/045	X2/045	42	34	80.85	< 0.001
	X1/116	X2/116	42	30	71.43	< 0.001
	X1/000	X1/100	42	17	40.48	-
	X2/000	X2/100	42	17	40.48	-
SKIM	000	100	84	34	40.48	-
	X1/000	X2/000	42	38	90.48	< 0.001
	X1/100	X2/100	42	37	88.10	< 0.001

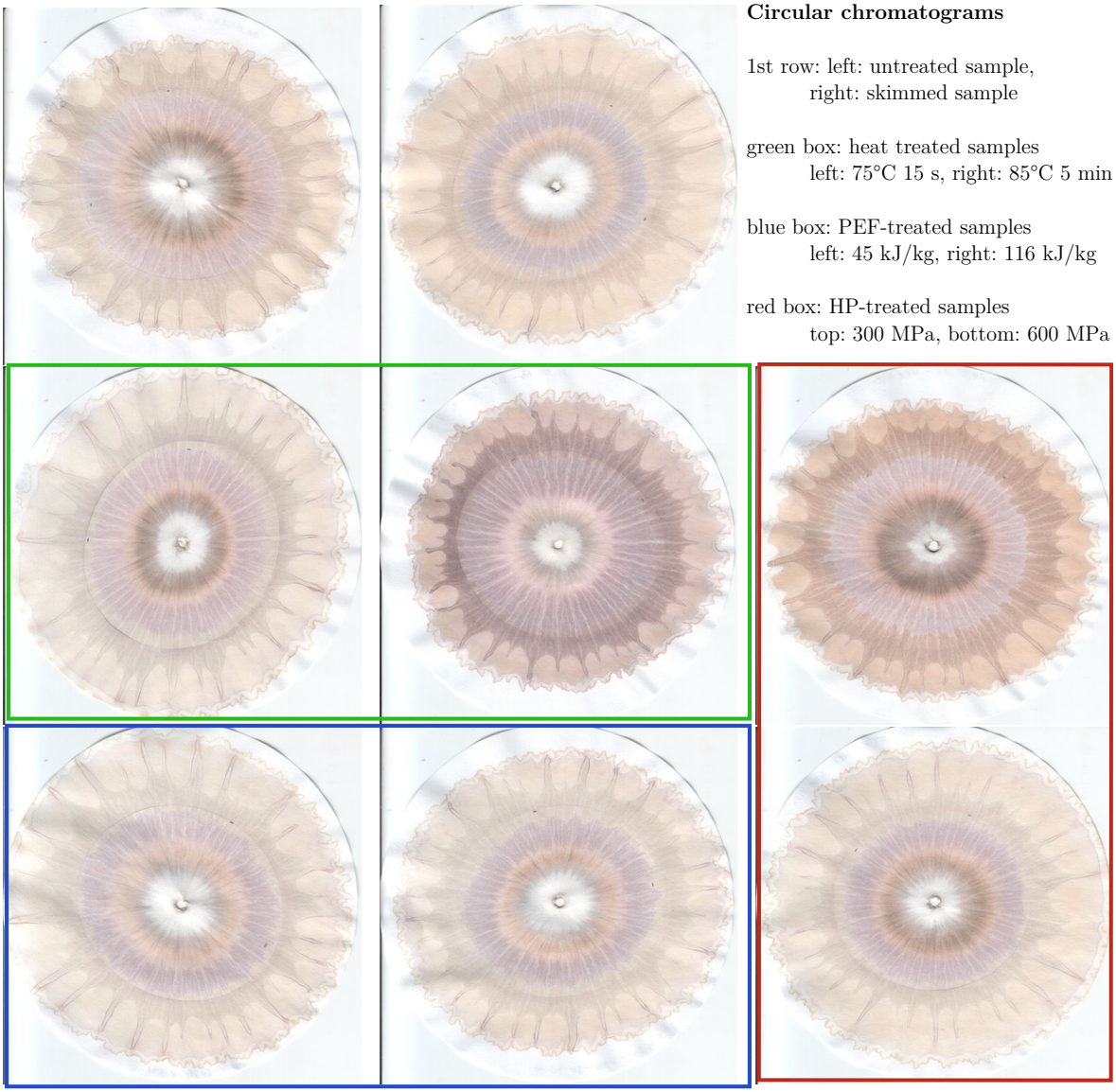


Figure 4.7: Selected circular chromatograms of the various treatments. Images are chosen from both repetitions each.

between the treated milk at low and high intensities for sample one, the chromatograms of both treatment-intensities must have appeared similar, but different from the second sample. As these results were not reproducible, the informative value is limited. In the few sources that are available from literature, however, some impacts of processing of milk on the patterns of circular chromatograms were found. Balzer-Graf and Gallmann (2000) found the chromatograms of the HP treated milk to have irregularities in the middle zone. Spikes were shorter and more angled than the chromatograms of untreated samples. The authors however used a different methodology and so results are not necessary comparable. Beckmann et al. (1993) observed changes in colour for pasteurized milk (85 °C for 8-10 s) in different rings as well as an impact of the heat treatment on the radiant rays that were less distinct. Also here, the methodology differed slightly. Both authors did not mention any repetition of experiments and thus

reproducibility of their results can only be guessed at.

Yet, differences found between the samples (repetitions) of all treatments were highly significant ($p < 0.001$ for all), thus showing that the reproducibility of the experiments was very poor. Indeed, when looking at the morphological variance in the pictures, it was observed that they differed enormously among the samples. Mostly, variations were observed in the colours of the rings (compare figure 4.7), indicating that the development of the colours was not entirely finished in some cases. The reason for this remains unclear as all chromatograms were developed under the same light conditions for the same amount of time as suggested by Beckmann et al. (1993). It might be possible that the age of the $AgNO_3$ solution plays a role. The solution was usually used for two subsequent experiments and thus was one week old at most.

Next to that, variations in colour and broadness of the rings in the inner zone occurred among the chromatograms of the two samples. In particular this applies to the white ring around the center (discussed below) and a dark-brown ring. No clear pattern in the variations could be observed for the latter. The broadness and intensity of colour differed among the repetitions but also among the four single chromatograms made from one subsample. Hence, no clear reason for this phenomenon could be spotted. Maybe slight variations in climate are the cause of colour differences. Also here, the usage of a climate controlled chamber might be a good solution. Possibly, temperature differences between the samples of the repeated experiments occurred at the start which led to a different flow behaviour of the compounds connected with changes in viscosity. The temperature of the samples was not monitored or controlled which is recommended for future experiments.

Link between the area of lowest optical density and protein content It is noticeable from figure 4.7 that the whitish area around the center of the circular chromatograms varies in size among the chromatograms of different samples. One can see in figure 4.8, there is a strong positive correlation between the concentration of total proteins in milk and these area of lowest optical density (whitish areas) around the center of the circular chromatograms. Therefore the findings of Knorr (1982) can be applied to milk and not only plant samples or protein solutions. However, one has to factor in that the standard deviations are relatively high, especially for higher protein concentrations. This is probably partially due to the fact that the edge of the whitish areas is not clearly developed enough and shows spikes. Also, flow inhibition plays a role. Besides the 'ruler method', Knorr (1982) used a soft laser scanning densitometer to assess the optical density. However, he obtained comparable results with both methods. To receive more precise results, chromatograms that show a flow inhibition should be sorted out and it must be clearly defined, how the areas will be determined when spikes occur. The influences of the treatments on the state of the milk proteins does not seem to have an effect on the size of the area of local density.

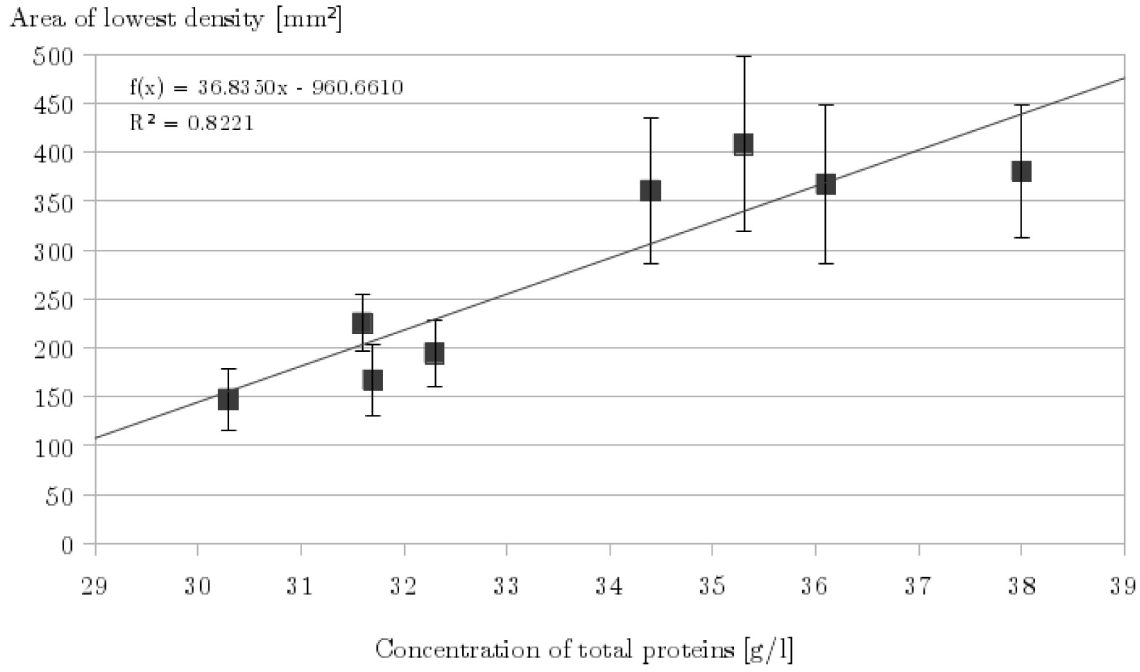


Figure 4.8: Link between the concentration of total proteins of the samples and the area of lowest density around the centers of the circular chromatograms.

To conclude, circular chromatography was unable in this case to detect any differences caused by the various thermal and non-thermal treatments. Furthermore, poor reproducibility is a great problem of the method. Yet, the method shows a potential to determine the protein-content of the samples as the whitish area around the center is linked with protein-concentration.

4.2.3 Biocrystallization results

The online image analysis test of the biocrystallization pictures was performed by 25 persons of which 24 % ended the test beforehand. The computerized texture and structure analysis of the biocrystallization method offered precise and objective tools to compare the effect of the different treatments on raw bovine milk. Below, the results of these tools are discussed. Hereby, also the effects of the different experimental days of each sample was taken into account. To avoid delay of the online image analysis this differentiation was ignored in the case of visual evaluation.

Results of the online visual evaluation Table 4.3 shows the outcomes of the online visual analysis which are discussed below. In figure 4.9 some example images of the different treatments are shown.

Results of the HP treatment Of all PFMs, the biocrystallization method seems to be the most promising one, especially in terms of reproducibility. Concerning the outcomes

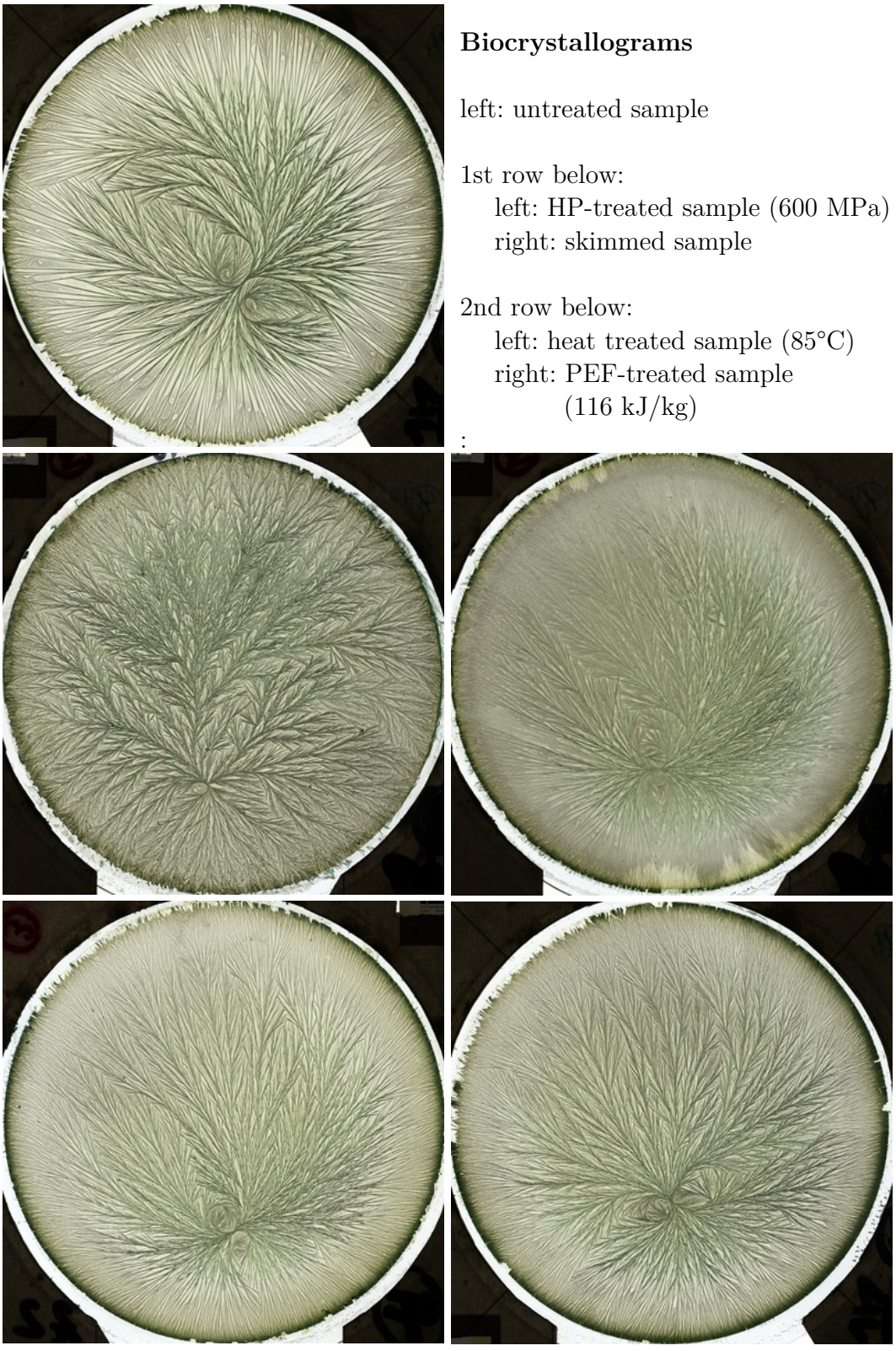
Table 4.3: Overview of the results of the online image analysis for the experiments conducted with the biocrystallization method. Abbreviations see table 4.1 on page 72

treatment	question * vs. *		total answers	correct answers	% correct answers	p-value
HP	X1/000	X1/600	50	32	64.00	< 0.001
	X2/000	X2/600	49	22	44.90	0.05
	000	600	99	54	54.55	< 0.001
	X1/000	X2/000	49	18	36.73	-
	X1/600	X2/600	49	18	36.73	-
HEAT	X1/000	X1/085	46	18	39.13	-
	X2/000	X2/085	46	19	41.30	-
	000	085	92	37	40.22	-
	X1/000	X2/000	46	13	28.26	-
	X1/085	X2/085	46	18	39.13	-
PEF	X1/000	X1/116	41	14	34.15	-
	X2/000	X2/116	41	18	43.90	-
	000	116	82	32	39.02	-
	X1/000	X2/000	40	15	37.50	-
	X1/116	X2/116	39	20	51.28	0.01
SKIM	X1/000	X1/100	38	28	73.68	< 0.001
	X2/000	X2/100	38	19	50.00	< 0.05
	000	100	76	47	61.84	< 0.001
	X1/000	X2/000	38	16	42.11	-
	X1/100	X2/100	38	15	39.47	-

of the online visual evaluation, significant changes were found comparing the untreated with the treated milk at 600 MPa. Hereby, the differences seemed to be bigger in the first ($p < 0.001$) than in the second sample ($p = 0.05$). In combination, the results were highly significant as well ($p < 0.001$). No significant differences were found between the crystallograms of different samples, both for treated and untreated milk. Thus, the results offer a good reproducibility which is probably also thanks to the fact that the climate controlled chamber was located in another climate controlled room, which limits the influence of outer fluctuations.

Generally speaking, untreated milk usually showed a good coordination from the center, long needles and less branching, whereas the coordination of the HP treated milk was rather poor, needles were shorter and more branching appeared, see figure 4.9. Additionally, it was noticeable that the biocrystallograms of the HP treated milk showed small 'dots' in between the crystal structure. These probably resembled flocculation in the solution that occurred after the milk had been added to the copper chloride and water solution. However, concerning the second experiment the flocculation did not occur on the first day, but only on the second.

A possible explanation for this might be fluctuations during transportation as a different transporting box was used for shipping whereby temperatures below the freez-



Biocrystallograms

left: untreated sample

1st row below:
left: HP-treated sample (600 MPa)
right: skimmed sample

2nd row below:
left: heat treated sample (85°C)
right: PEF-treated sample
(116 kJ/kg)
:

Figure 4.9: Selected biocrystallograms of the various treatments.

ing point of milk could have occurred which led to further protein denaturation, also called cold denaturation. For example, de Jongh et al. (2001) observed conformational changes in β-lactoglobulin for temperatures around -20 °C, however, cold denaturation is theoretically already possible at temperatures below 0 °C (Pühse, 2009). Mulsow

(2008) discussed that these conformational changes include the presence of hydrophobic areas on the protein surface whereby alterations in the solubility behaviour and the stability of the protein are to be expected. Saadati and Razzaghi (2012) found that the sensitivity towards cold denaturation of β-lactoglobulin solutions is higher in the presence of α-caseins. Literature suggests that cold denaturation can also occur for caseins. For example, Farrell Jr et al. (2001) showed that already at 2-4°C monomers of β-casein are less structured. How these findings apply to the complex system of milk is however only poorly discussed in literature. Töpel (2004, p. 693) solely mentions that freezing of milk leads to poorer quality.

Possibly, also contact of the cupric chloride solution and agitation of the sample mixtures for a longer time (> 30 min) led to earlier flocculation. The energy input during agitation might have been bigger than the repulsion between the interacting whey proteins and casein (sub-)micelles which could have led to the formation of bigger aggregates and therefore flocculation. Agitation time on the first day for the second sample could have been shorter than for the other days and thus the energy input might have been too low to cause flocculation. Unfortunately, the agitation time was not monitored. Furthermore, the flocculation effect was not observed in other untreated or heat-, PEF treated or skimmed samples as, less interaction occurred between caseins and whey proteins during the treatments. Thus, in those cases, the energy input during agitation might have been too low to cause the formation of bigger aggregates. If agitation would have been performed for a longer time, it could have been possible that flocculation also occurred for the other samples of other treatments.

The biocrystallograms of HP treated milk, where the proteins did not aggregate, remind one more of the images of untreated milk with longer needles and less branching. In addition, these branches tended to remain straight, whereas the branches of the images with flocculation tended to describe curves.

Although these structural and morphological alterations did occur, no significant variations were found by the testers when comparing the two samples in the online image analysis test. This is probably due to the fact that characteristics only digressed for the first but not for the second day of the second sample. As the two days were grouped together for each sample, roughly half of the pictures showed a 'flocculation structure' and resembled the crystallograms of the first sample.

In the generally limited literature available, only Balzer-Graf and Gallmann (2000) discussed the influences of HP-processing of milk on the crystal patterns. They observed changes in the outer zone of the crystallograms. Hereby, HP treated samples showed less thin branching in this zone and the needles seemed to be thinner which was not observed in the experiments conducted in this thesis. However, the exact methodology Balzer-Graf and Gallmann (2000) used is unknown and thus comparability is only limited. Selawry and Selawry (1957) surmised before that different proteins present in the samples are responsible for the difference in crystal formation and the

angles of branches and needles. From the results present here it becomes evident that not only the type but also the state of proteins present in the samples has a major influence of pattern formation.

To conclude, the HP treated samples were sensitive to the $CuCl_2$ solution as flocculation occurred during agitation. This led to the formation of small 'dots' in between the crystal structure, shorter needles and higher degree of branching. HP treated samples were differentiated significantly from untreated samples.

Results of the heat treatment Apparently, the testers were not able to distinguish the heat treated (85 °C) from the untreated milk as no outcomes were substantial, compare table 4.3 on page 82. Moreover, no differences were observed between the biocrystallograms of the two repetitions. So, the results were at least reproducible, albeit no morphological changes were discovered by the test subjects. Online visual evaluation failed to detect protein denaturation in the heat treated milk.

However, when looking at the example images in figure 4.9 one can see differences in the crystal structures of the crystallograms derived from the untreated compared to the heat treated milk. The crystal structure of the heat treated milk appears more dense. The needles are shorter and thinner there is slightly more branching. Branches seem to describe curves. In the outer zone where only single needles are present, the image appears less 'clear' for the heat treated milk. This phenomenon is probably based on the higher density of needles in this zone. Wohlers et al. (2007) described the crystallization patterns of raw and UHT treated milk. They found an increased amount of thinner needles in the crystallograms of the UHT treated samples. The branches were more bent for the UHT treated milk whereas they are more straight for the untreated one. Also, the outer zone of the crystallograms appeared more clear. These results are in line with the findings of this thesis.

Unlike the HP treated samples, no flocculation occurred during sample preparation and so the typical 'flocculation structure' like with the pictures of HP treated milk was not observed. This indicates that the HP-typical flocculation and the change in 'gestalt' are mainly caused by formation of large aggregates during agitation which can be linked to the disruption of the caseins, their interaction with the whey proteins and their sensitivity towards the $CuCl_2$ solution.

The inability to identify divergences in the crystal structures might be attributed to the fact that the two above-mentioned 'gestalts' can be found to some extent in both the untreated and the heat treated samples. Generally, the 'gestalts' of the crystallograms of the heat treated and untreated samples change with the nucleation time: early crystallized images look different than middle or late crystallized ones. In this evaluation, the images with a late nucleation time (above 15 hours) were already sorted out. However, it might have been interesting to group images according to the starting

time of the crystallization. It can be surmised that the test subject would have been able to identify the images more precisely. On the other hand, one can argue that all arisen changes could be caused not by the sample modifications but by subtle outer and inner fluctuations, such as the temperature, humidity or concentrations which have an impact on the nucleation time and thus also on the forming of typical 'gestalts'.

In summary, slight differences were observed between the crystal structure of the heat treated compared to the untreated samples: e.g. shorter and thinner needles. These differences were not detected in the online analysis test, which is probably because the influence of the nucleation time outweighs the effect of the heat treatment.

Results of the PEF treatment With PEF treatment, again, the test subjects were not able to identify changes in the crystal structure for both samples except in one case. This indicates that the state of proteins in the milk plays a role in pattern formation, as significant changes were observed for HP- but not for PEF treated samples. Significant variations were found for the PEF treated milk between the two samples ($p = 0.01$). It remains unclear why these significant differences were found between the crystallograms of the repeated experiments as the morphological features seemed alike. Variations might be caused by divergences concerning the nucleation time caused by slight differences in the chamber climate between the two experiments.

However, when looking at the morphology, the case is similar to that of the heat treated milk: About the same alterations were found in the biocrystallograms of the untreated and the PEF treated milk, compare figure 4.9. These findings are interesting, as no changes in the structure of th milk proteins were observed via HPLC and no changes in the chromatograms were detected by the test subjects. It can also be guessed that other structural changes which were not detected in these experiments might be the cause of the changes in the 'gestalts' of the images. For example, the denaturation of certain enzymes or the inactivation of micro-organisms might play a role in pattern formation. It is possible that the differences found by the (trained) author were not visible for the untrained test subjects. Furthermore, the influence of the nucleation time could have outweighed the impact of the PEF treatment.

To sum up, the biocrystallograms of the PEF treated samples resembled the ones obtained with the heat treated samples, however the differences to the untreated samples were not confirmed by the test panel. Again it is likely that the nucleation time outweighed the influence of the PEF treatment of pattern formation.

Results of skimming Crystallograms of the skimmed milk generally showed a haze behind the crystal structure which was most visible in the outer zone where the crystal density is lower. Next to that, some clear transparent spots appeared on the edge on

most crystallograms of skimmed milk. Crystal formation did not seem to be influenced on these spots, in fact the clear transparent areas resembled the ones found with the untreated milk. This suggests that the milk fat might have an influence on the area in between the needles and is responsible for the clear transparent appearance. Lack of milk fat therefore might have led to the formation of a haze. The clear transparent spots of the skimmed samples might represent the areas where residual fat gathered. Why this happened mostly in the outer zone remains mainly unclear. Potentially, creaming occurred during the long crystallization times. As discussed in section 2.4.3, self-organization processes might have taken place in the form of one big Bénard-like cell. The 'flow' on the surface of the cell points towards the edge. Light milk fat swimming on the surface of the sample mixture was possibly carried along with the subtle flow towards the edge of the cell and so the clear transparent spots mainly occurred towards the edge of the plate.

Similar to all other treatments discussed before, crystal needles of the skimmed milk were shorter and more branching occurred with the branches describing curves, compare figure 4.9.

Like for the HP treated samples, the test persons were able to distinguish between raw and skimmed milk very well. Hereby, significance proved to be higher for the first ($p < 0.001$) than for the second sample ($p < 0.05$). These findings are interesting as relatively more clear transparent spots occurred in the biocrystallograms of the second sample. It can be guessed that randomly more crystallograms were picked that lacked those spots and that the subjects did not detect the haze that was visible in the crystallograms of the skimmed milk. Repetitions of the experiments were not significantly different and thus the results are shown to be reproducible. It was proven that biocrystallization is sensitive to other structural changes in milk besides protein denaturation.

To conclude, skimming of milk led to the occurrence of a haze visible in the biocrystallograms, next to clear transparent spots which are probably linked to residual milk fat. Needles were shorter and more branching occurred. Online image analysis confirmed the outcomes.

Results of the texture and structure analysis of the biocrystallograms From both computerized analyses, structure analysis gave the most significant results, whereas no differences were found by the texture analysis. Many hundred of different graphs were created during the analysis. Only a few of the graphs which show the most meaningful results are discussed below. Unlike the online image analysis test, also the effects of the experimental days were taken into account as crystallization was carried out twice at a two days interval with the same sample. No significant results were obtained by texture analysis for all analyses and will therefore not be mentioned further. The results are

discussed in detail for the HP treated samples. Subsequently, only main conclusions will be presented.

Results of the HP treatment HP treatment of milk had a noticeable effect on the crystallization pattern, as we can see in the principle component analysis (PCA) score-plots from the structure analysis category length at ROI 40% (see figure 4.10). The result was reproducible as they could be confirmed with both samples (same figure). PCA results were not consistent for the two sample repetitions for the structure analysis category width (data not shown). Thus, one can conclude that HP treatment seems to have an effect on the length of the crystal-needles, but not on their width, which confirms the findings of the visual evaluation.

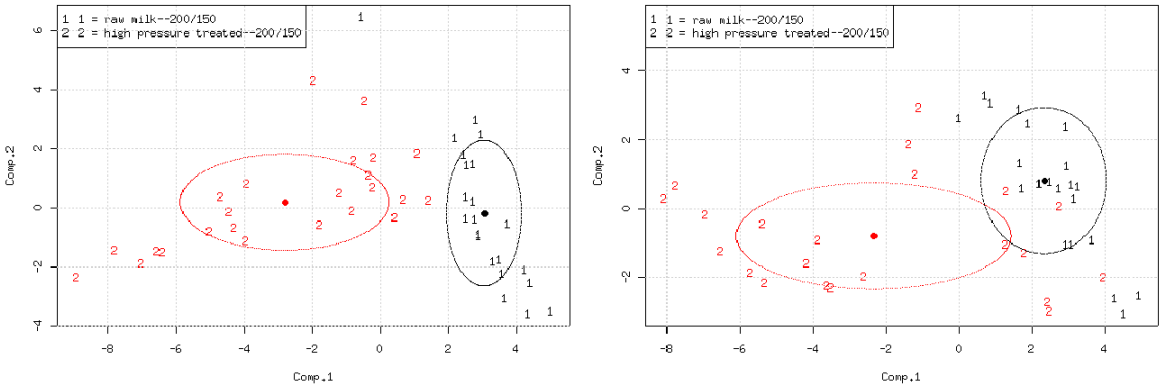


Figure 4.10: PCA score-plots of ROI 40 % of the structure analysis variables for the category length. Dot: mean, circle: standard deviation. Black: untreated milk, red: HP treated milk. Left: sample one, right: sample two (repetition).

The same conclusions can be draws from figure 4.11. LME-ANOVA based on the structure analysis category-length variables showed a significant difference between the crystallization patterns of the untreated and the HP treated milk for each single ROI (figure 4.11, left, black boxes, $p < 0.01$). Additionally, a day-effect[1] was found (same graph, red boxes, $p < 0.05$ for ROI > 20 %). The significant day-effect proposes that the length of the crystals differed among the day-repetitions. This might be due to aging of the milk or slight differences in the climate. Also, flocculation might play a role. The findings could be repeated with another sample figure 4.11, right, black boxes, $p < 0.01$). In this case, the day-effect was only significant for ROIs > 20 % (same graph, red boxes, $p < 0.05$ for ROI 30-40 % and $p < 0.01$ for ROI $\geq$50 %).

For both samples, also an interaction between treatment and day was found for ROI > 20 % (green boxes $p < 0.05$). This suggests that the day-effect artificially caused the treatments to differ significantly more from each other and therefore it is unknown from these graphs whether the significant treatment-effect is valid. The day

[1] effect between the two repetitions conducted with the same sample

effect was stronger for the second than for the first sample, especially at ROI > 50 % and was maybe also partly responsible for the observed interactions between treatment and day.

As discussed earlier, flocculation of the sample mixtures only occurred on the second day of the second experiment which was also visible in the crystallization patterns: the crystallization patterns of the HP treated milk of the first day, second sample resembled the patterns of the untreated milk more than the HP treated ones of the second day and the first sample. Differentiation was less strongly significant for the second ($p < 0.05$) than for the first sample ($p < 0.001$). This might have caused the occurrence of a significant day effect.

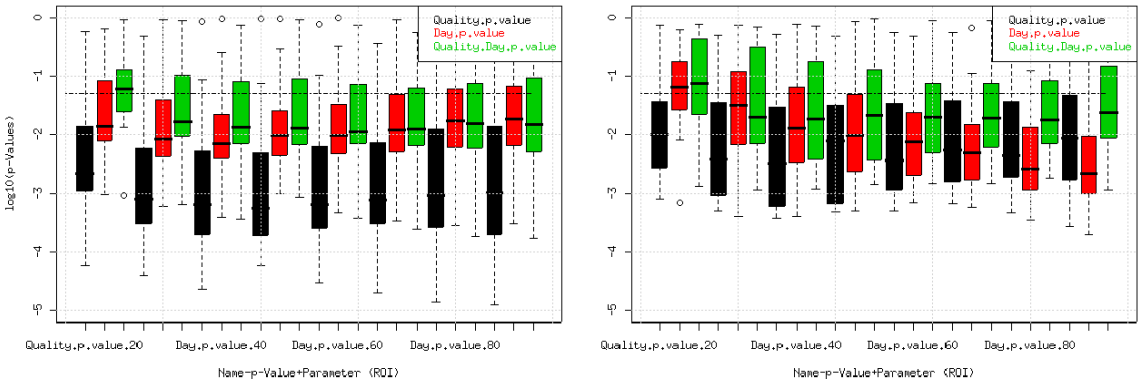


Figure 4.11: Log(p)-values for the difference between the crystallization patterns of the untreated and HP treated milk (y-axis) over the analyzed ROI (x-axis) for all structure analysis variables of the category length. Black: effect of treatment, red: effect of day, green: interactions of treatment and day. Left: sample one, right: sample two. Dashed horizontal line depicts p = 0.05.

In the box-plots of figure 4.12 is shown which effect HP has on the needle structure: The needles become shorter as the crystallization patterns of the HP treated milk exhibit fewer long crystal fragments (length variable L220). As the results of figure 4.10 and figure 4.12 both point into the same direction, it is likely that the poor results shown in figure 4.11, right graph are probably due to a lack of statistical power and more repetitions might have solved the problem of the interactions between treatment and day. The effect of the (lacking) flocculation is not visible in figure 4.12, left graph. Therefore, the length of the needles for HP treated samples were not influenced by the presence or absence of flocculation in the sample mixture. The effect of the disruption of the caseins and their interaction with whey proteins caused by HP treatment seems to be stronger than the damage caused by the $CuCl_2$-solution and agitation. Therefore, it can be surmised that the size of casein micelles had an influence on the needle formation.

As mentioned earlier in section 2.4, crystallograms with a late nucleation time usually show a different crystal pattern or 'gestalt' than than those with an earlier nu-

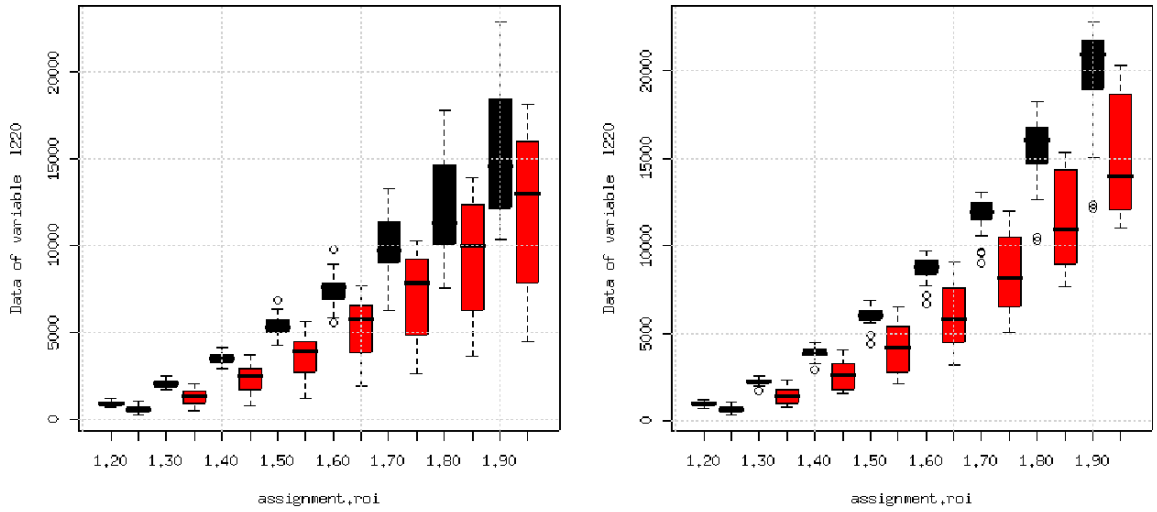


Figure 4.12: Boxplots for the L220 length variable. Y-axis: number of crystals found, x-axis: ROI 20-90 %. Black: crystallization patterns of untreated milk, red: HP treated milk. Left: sample one, right: sample two.

cleation time. For example, multiple centers might occur for late crystallized images. To determine the proportion of the variance of the structure variables category-length that is predictable from the nucleation time, the adjusted coefficient of determination ($\bar{R}^2$) was plotted against the ROI (see figure 4.13). A ROI-dependent correlation was found for the crystallization patterns, however, only for the untreated milk sample. For example, for larger ROIs ($\geq$ 80 %) approximately 70 % of the total variation in the combined structure variables category length could be explained by the linear relationship with the nucleation time. Skimming of the milk disrupted this correlation, which was found for both analyzed samples (compare left and right graph), which means that the influence of the HP treatment here outweighed the impact of the nucleation time.. The disruption effect was stronger for the first sample. It is likely that this again is caused by the different behaviour of the two sample mixtures of the different experimental days.

To conclude, structure analysis showed that HP treatment of milk led to shorter needles which can be associated with the changes in the protein fractions. Hereby, HP treatment outweighs the influence of the nucleation time. This confirms the findings of the visual evaluation.

Results of skimming As one can see in figures 4.14 and 4.15, skimming of milk shows an noticeable effect on the length and width of the needles. Hereby, the results were always evident for both samples examined and consistent for all experimental days. Like HP treatment, fewer long needles were found, compare figure 4.16, right graph. Additionally, more short needles were detected, compare figure 4.16, left graph. From this can be concluded that, next to the state of proteins, also the fat content of the

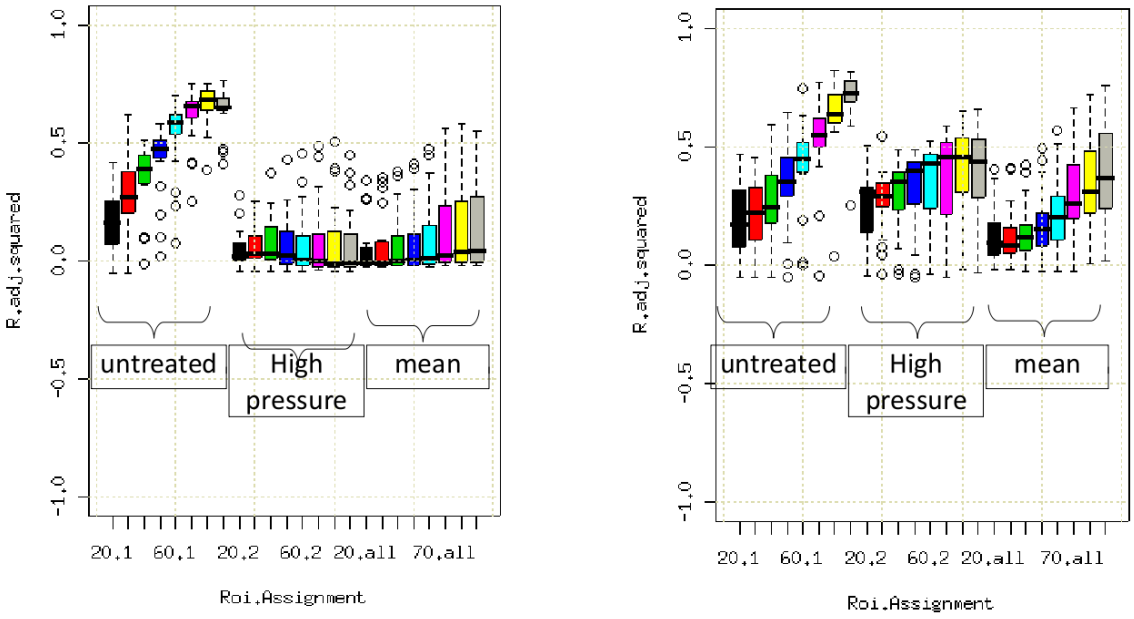


Figure 4.13: $\bar{R}^2$ over the analyzed ROI for the crystallization patterns of the untreated and HP treated milk. Left sample one, right sample two.

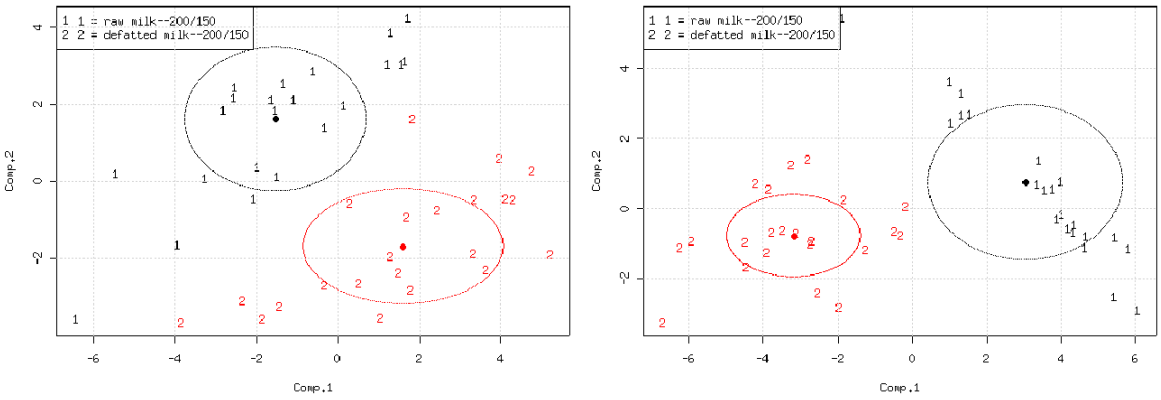


Figure 4.14: PCA score-plot of ROI 80 % of the structure analysis variables for the categories width (left) and length (right) for sample one. Black: crystallization patterns of untreated milk, red: skimmed milk.

milk samples has an influence on crystal formation.

Similar to HP treatment, the effect of skimming hereby outweighs the effect of the nucleation time on pattern formation, compare figure 4.17. The results of the visual evaluation can be confirmed. Again, the effect was higher for the first than for the second sample. As HP treated and skimmed milk samples were analyzed together, a common unknown factor might have caused this phenomenon, for example mistakes during sample preparation or changes in the climate of the chamber between the two samples as well as between the two experimental days of the second sample. One can conclude that the effect of skimming on the pattern formation in the crystallograms outweighs the influence of the nucleation time and this could be detected by visual evaluation as well as by structure analysis.

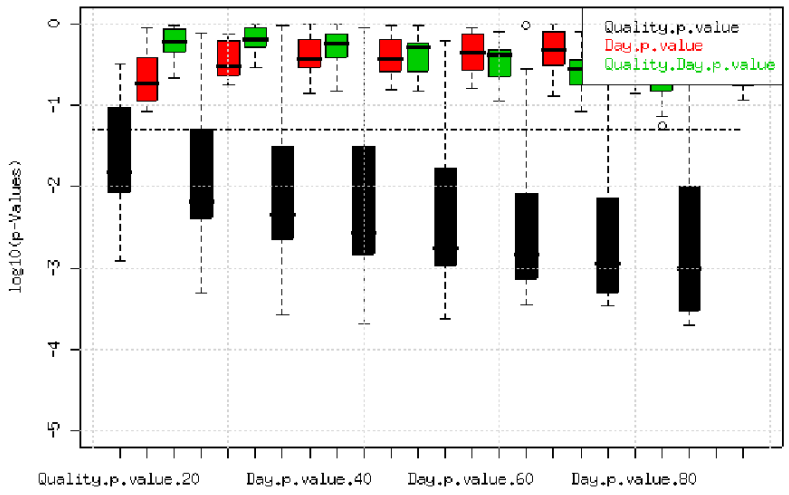


Figure 4.15: Log(p)-value for the difference between the crystallization patterns of the untreated and skimmed milk over the analyzed ROI for all structure analysis category-length variables of sample one. For explanation see figure 4.11.

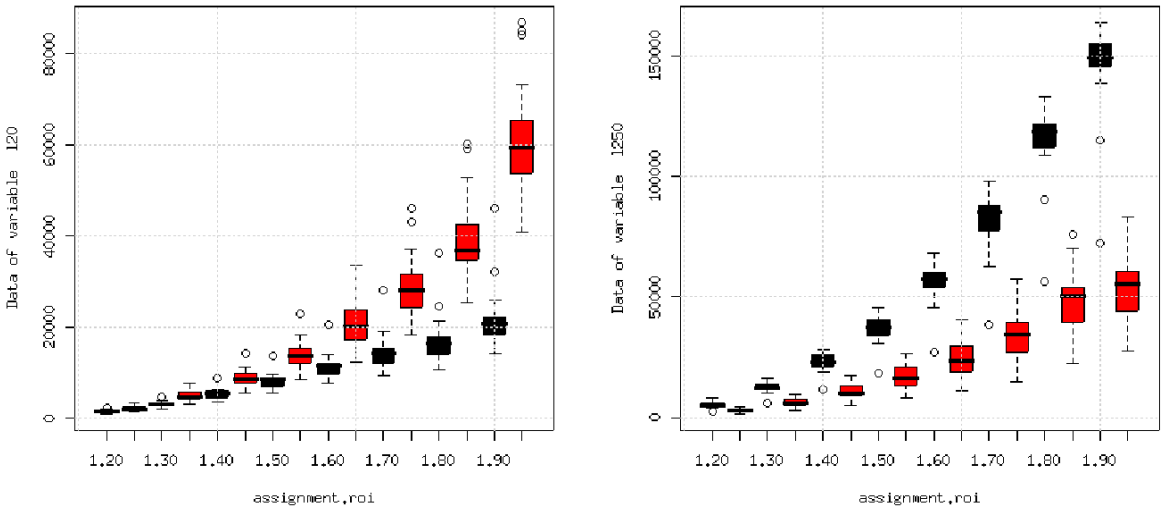


Figure 4.16: Boxplots for two different length categories for sample one (left: short crystal fragments L20, right: long crystal fragments L250). Y-axis: number of crystals found, x-axis: ROI 20-90 %. Black: untreated milk, red: skimmed milk.

In summary, skimming of milk led to the formation of shorter and thinner needles as became evident with structure analysis. Hereby, the effect of the treatment on pattern formation was stronger than the influence of the nucleation time. The results confirm the findings of the visual evaluation. Therefore it was shown that proteins are not the only influencing factor in pattern formation of the biocrystallograms.

Comparison of HP treatment and skimming Skimming and HP treatment on raw milk were evaluated simultaneously with the same samples and therefore it was possible

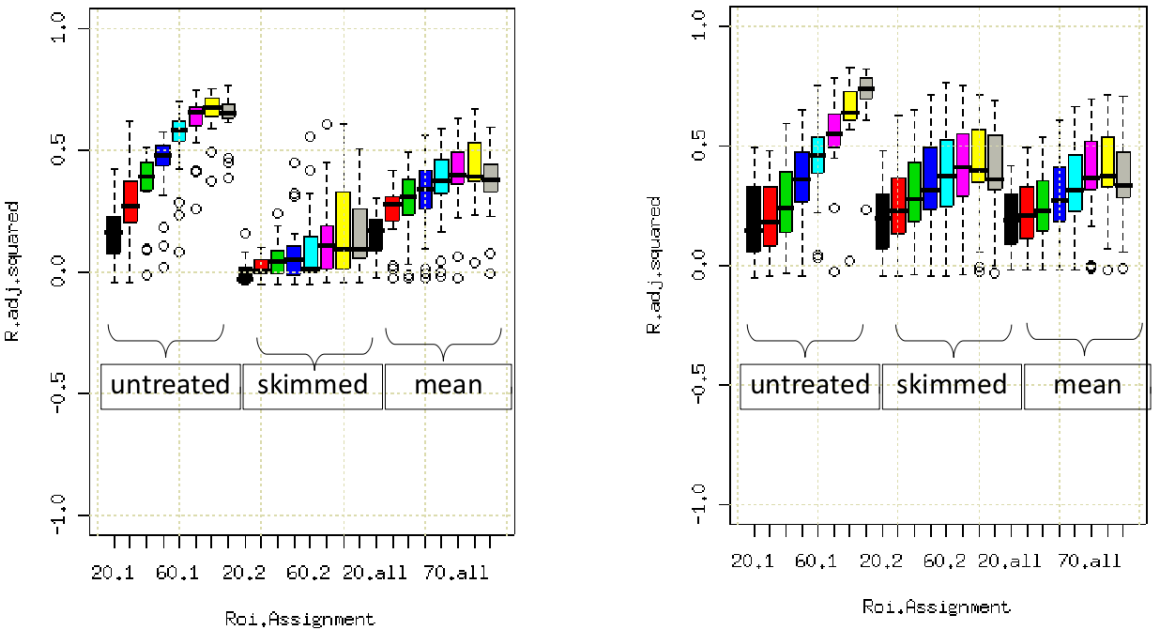


Figure 4.17: $\bar{R}^2$ versus the analyzed ROI for the crystallization patterns of the untreated and skimmed milk. Left: sample one, right: sample two.

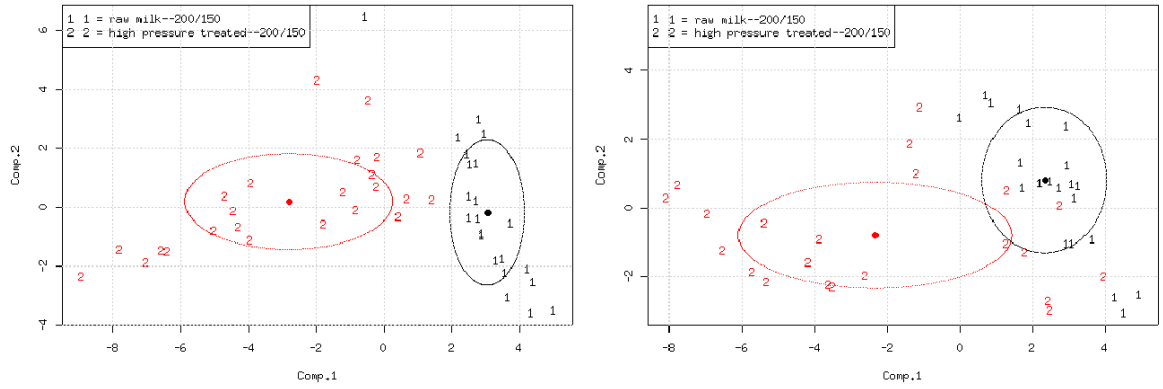


Figure 4.18: PCA score-plots ROI 40 % of the structure analysis variables for the category width. Black: HP treated milk, red: skimmed milk. Left: sample one, right: sample two.

to determine whether the two treatments had a similar effect on the crystallization patterns. Although the effects of HP treatment and skimming seemed to be similar when examined separately, figures 4.18, 4.19 and 4.20 illustrate that both treatments had dissimilar effects on crystal formation.

Hereby, the crystals formed with skimmed milk exhibit more wide needles than for HP treated milk, compare figure 4.20. It can be surmised that the presence of fat suppressed crystallization processes that lead to the thickening of needles but favoured linear growth.

No difference in needle length was encountered, suggesting that both treatments led to a comparable shortening of crystal needles. This is interesting, as it clearly shows that not only changes in the protein fraction of milk can be made visible with

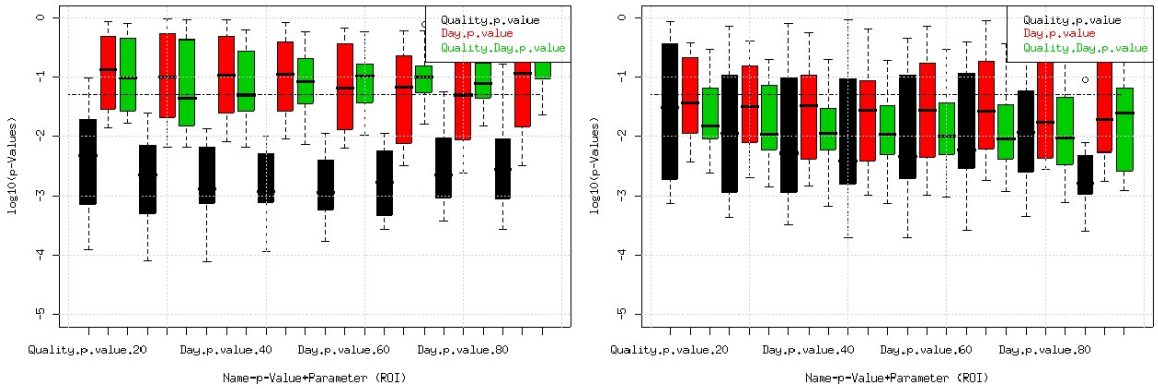


Figure 4.19: log(p)-values for the difference between the crystallization patterns of HP treated and skimmed milk versus the analyzed ROI for the combined width variables. Left: sample one, right: sample two. For explanation see figure 4.11.

the biocrystallization, but also other structural changes have an influence on pattern formation. The exact mechanism of crystal growth is left unclear. Both proteins and fat present large fractions of the portion of dry matter in milk and play crucial parts in the mechanism of pattern formation. Thus when one part is altered, the patterns exhibit changes.

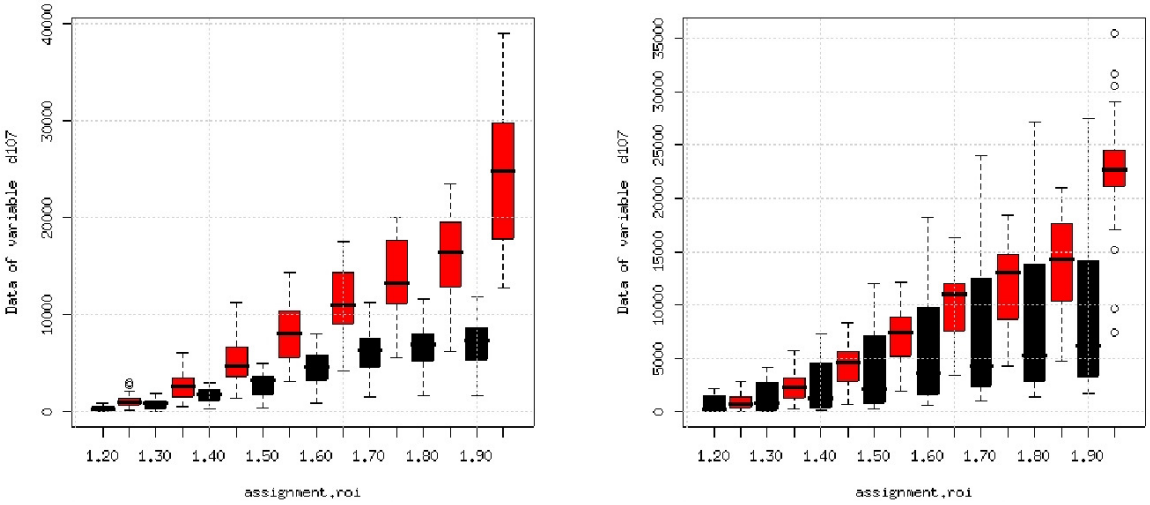


Figure 4.20: Box-plots for the D107 width variable. Y-axis: number of crystals found, x-axis: ROI 20-90 %. Black: HP treated milk, red: skimmed milk. Left: sample one, right: sample two.

As is visible form figures 4.18, 4.19 and 4.20, the differences were less strong for the second sample which was also observed with visual evaluation. Again, it is likely that the different behaviour of the sample preparations for the second sample (flocculation) plays a role, as a day effect and interaction between treatment and day were found, compare figure 4.19.

To conclude, it became evident with structure analysis that HP treatment and skim-

ming had dissimilar effects on the crystallization structure. Whereas both treatments led to the formation of shorter needles, the crystallograms of skimmed milk exhibited thinner crystal needles. This illustrates that both fat and proteins play a role in pattern formation of the crystal structures.

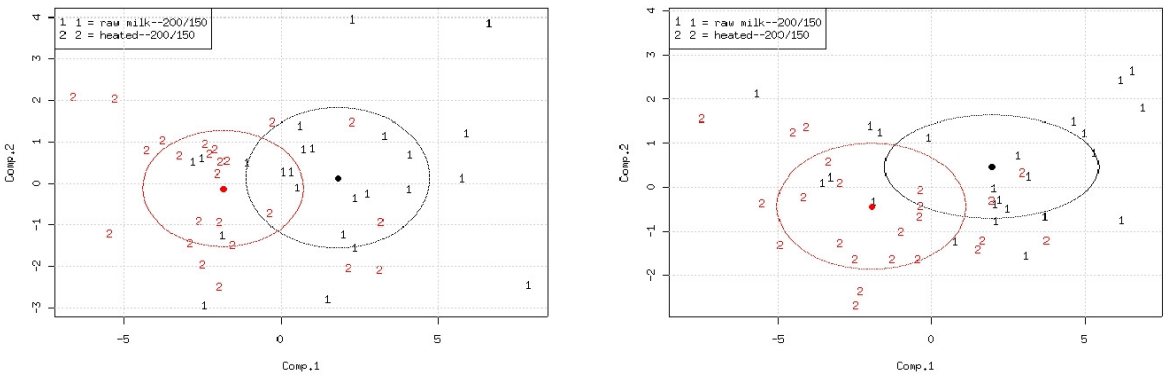


Figure 4.21: PCA score-plot ROI 40 % of the structure analysis variables for the categories width (left) and length (right) of sample one. Black: untreated milk, red: heat treated milk.

Results of the heat treatment Compared to HP treatment and skimming, heat treatment only shows a moderate effect on the width and length of the crystal needles, compare figures 4.21 and 4.22. Furthermore, with PCA and LME-ANOVA, the result could not be confirmed with a second sample (data not shown).

Yet, when the number of needles is plotted over the ROIs, it becomes visible that the crystallized heat treated milk exhibited less wide crystal fragments in combination with fewer longer needles compared to the crystallization patterns of untreated milk, see figure 4.23. These differences were found for both analyzed sample-pairs. This indicates that the non-significant differences found for PCA and LME-ANOVA of sample two was probably again caused by a deficit in statistical power (too few repetitions).

Additionally, figure 4.24 illustrates that the influence of the nucleation time on pattern formation was bigger than the effect of the heat treatment which was found for both sample-pairs. This is in line with the findings of visual evaluation where the subtle differences were detected by the author but not by the untrained test subjects.

Compared to HP treatment, little structural changes were observed for the protein fractions of heat treated milk: whey protein denaturation and their interactions with caseins. No change in size was detected for the casein micelles. The influence of these structural changes was probably too small to show a clear effect on the crystallization pattern as observed with HP treatment and skimming, where relatively big structural changes in the milk occurred. Interestingly, heat treatment led to thinning of the crystal needles which was not observed for HP treated milk, although here, also whey denaturation and interaction with caseins was encountered. Furthermore, the denaturation process of β-lactoglobulin for heat treatment follows the same mechanism

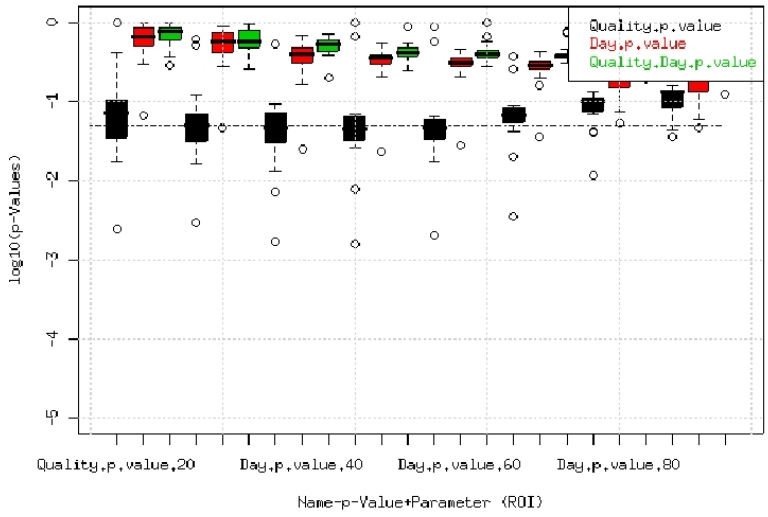


Figure 4.22: log(p)-value for the difference between the crystallization patterns of the untreated and heat treated milk over the analyzed ROI for the combined length variables, sample one. For explanation see figure 4.11.

as for HP treatment: the uncovering of thiol-groups and the subsequent reaction with either caseins, other molecules ofβ-lactoglobulin or α-lactalbumin (Hinrichs, 2000).

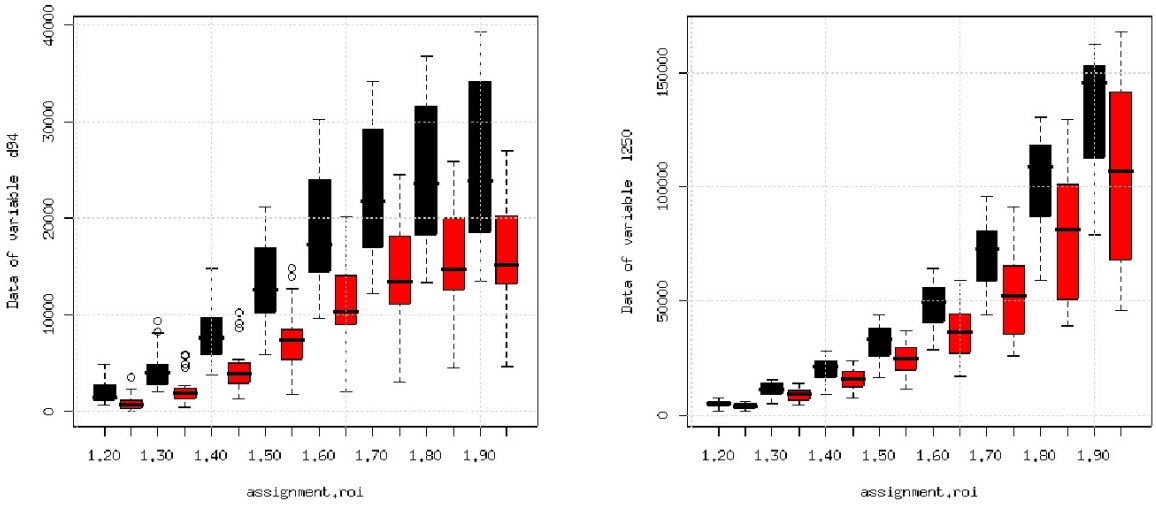


Figure 4.23: Boxplots for width variable D94 (left) and length variable L250 (right) versus the analyzed ROI of sample one. Y-axis: number of crystals found, x-axis: ROI 20-90 %. Black: untreated milk, red: heat treated.

Hence, if whey protein denaturation leads to a decrease in width of the needles for both treatments, the reason for the unchanged width of the needles for the HP treated milk must have something to do with the disruption of the caseins and/or flocculation.

In summary, structure analysis showed that heat treatment of milk led to the formation of thinner and shorter needles, which is probably influenced by whey protein denaturation. However, the influence of the nucleation time on pattern formation was found to be higher than the effect of heat.

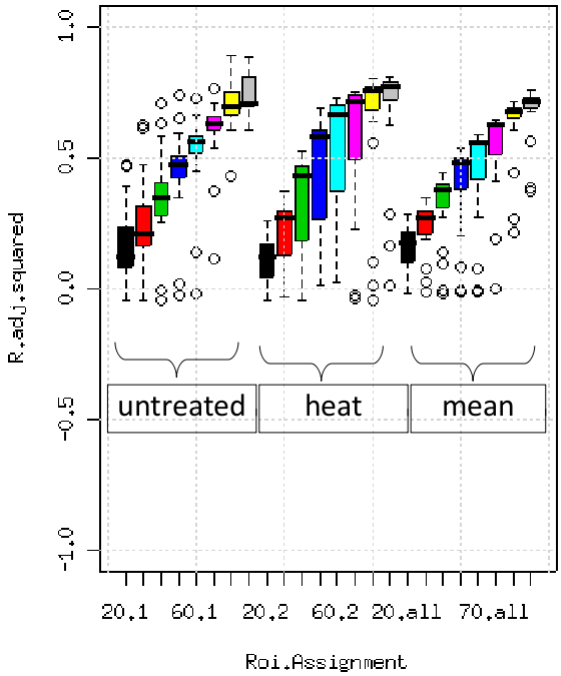


Figure 4.24: $\bar{R}^2$ versus the analyzed ROI (x-axis) for the crystallization patterns of the untreated and heat treated milk for sample one.

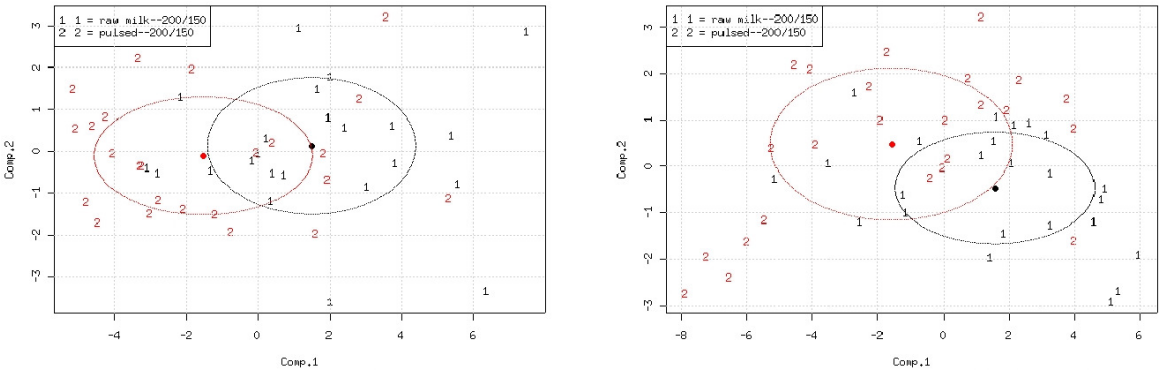


Figure 4.25: PCA score-plot ROI 40 % and ROI 30 % of the structure analysis variables for the categories width (left) and length (right), respectively of sample one. Black: untreated milk, red: PEF treated milk.

Results of the PEF treatment Similar to heat treatment, PEF treatment was shown to have an effect on the width and length of the crystal needles, compare figures 4.25 and 4.26. Again, result were not confirmed by the second sample (data not shown). This is different when looking at the number of needle-fragment over ROI, compare figure 4.27. Here, less wide and less long needles were observed for both analyzed sample-pairs. This indicates that, again, the amount of plates was too low to show significant effects for PCA and LME-ANOVA for sample two. Like heat treatment, the influence of the nucleation time on pattern formation was bigger than the effect of PEF treatment which is probably the reason why the test subjects were not able to detect the fine differences whereas the author was able to do so.

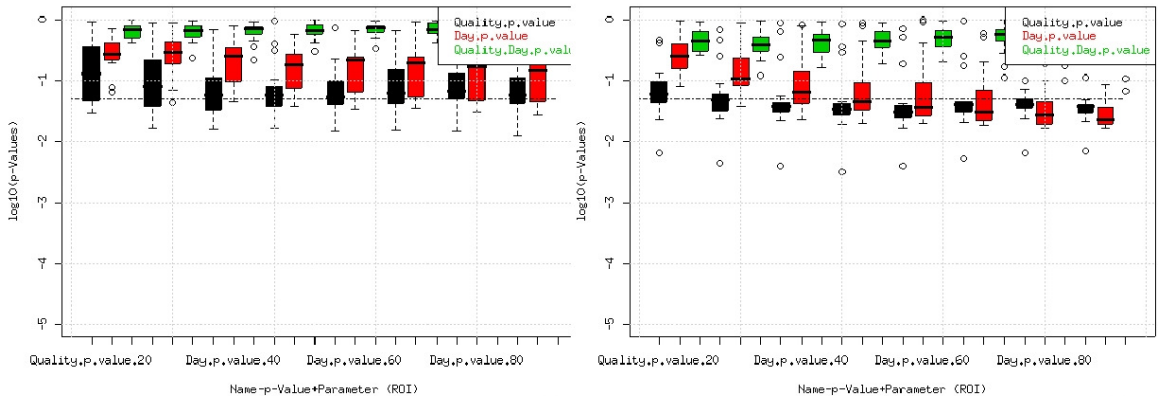


Figure 4.26: log(p)-value for the difference between the crystallization patterns of untreated and PEF treated milk over the analyzed ROI for the combined width (left) and length (right) variables for sample one. Explanation see figure 4.11.

As no changes in both protein fractions, caseins and whey proteins were detected via HPLC the result are interesting. Although not as significant as for HP treatment and skimming, it was shown that the influence of PEF led to changes in the crystal structure. It can only be guessed which changes in the milk caused the subtle alterations of the crystal structure. Above all, micro-organisms and enzymes are inactivated by PEF, but also some vitamins are destroyed, compare section 2.2.4.

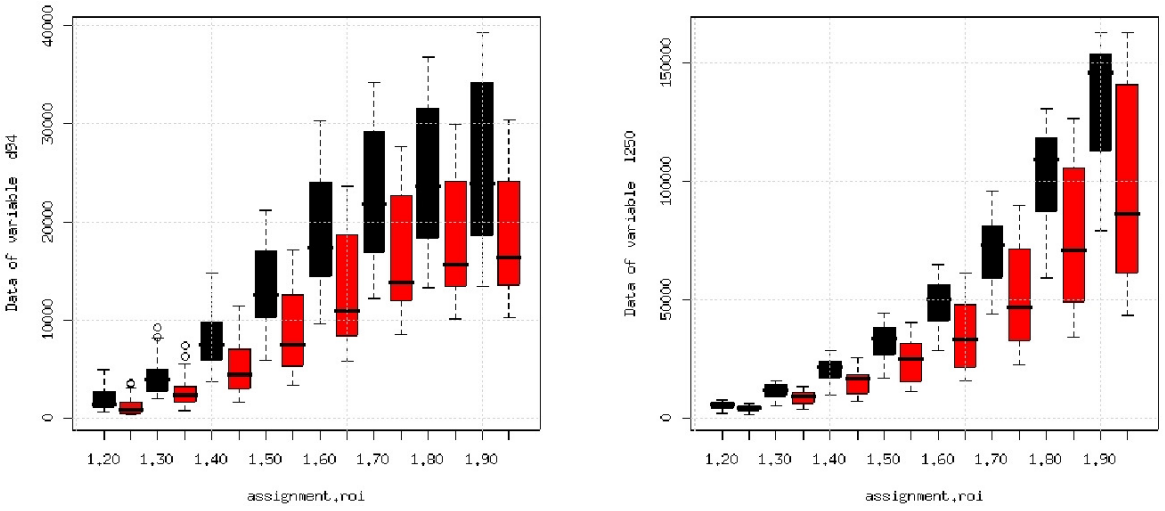


Figure 4.27: Boxplots for width variable D94 (left) and length variable L250 (right) versus the analyzed ROI for sample one. Y-axis: number of crystals found, x-axis: ROI 20-90 %. Black: untreated milk, red: PEF treated.

As enzymes are proteins as well it is likely that their inactivation plays a role. For milk, mainly lipase and peroxidase are affected (Grahl and Märkl, 1996). However, as the amount of enzymes in milk is relatively small compared to caseins and whey proteins. For example, the concentration of lipase is in the range of 1-2 ml/l (Töpel, 2004). Enzymes are catalysts for very different kinds of reactions. It is possible that

they are also involved in pattern formation. Unlike HP treatment the effect of PEF on substances in milk are not thoroughly examined. Hence, unknown and undetected changes might also have influence on pattern formation in the biocrystallograms

To conclude, structure analysis showed that PEF treatment of milk led to the formation of less long and less wide crystal fragments which might be attributed to the inactivation of enzymes or micro-organisms in milk. These changes were however outweighed by the influence of the nucleation time.

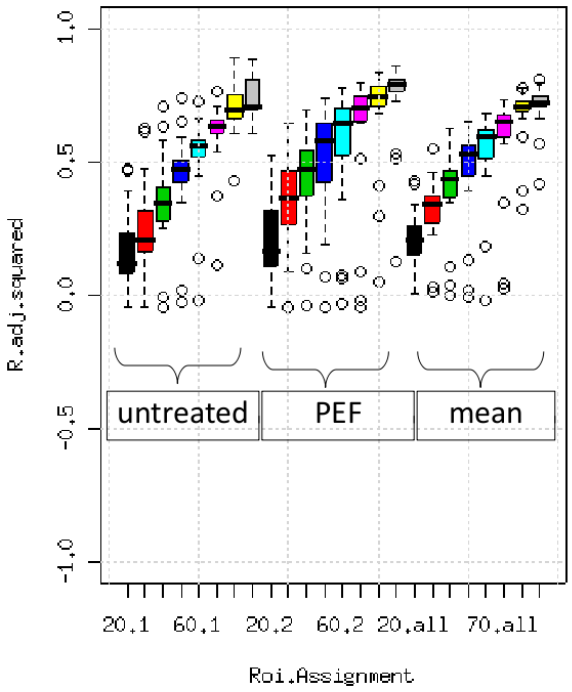


Figure 4.28: $\bar{R}^2$ versus the analyzed ROI (x-axis) for the crystallization patterns of untreated and PEF treated milk, sample one.

Comparison of heat- and PEF treatment Heating and PEF treatment of milk were evaluated simultaneously with the same samples. Therefore, it was possible to determine whether the two treatments had a similar effect on the crystallization patterns. PCA with both structure analysis categories (width and length) gave no differentiation (data not shown). This was consistent with the finding noted above that both the heat and the PEF treatment exhibit less wide crystal fragments in combination with fewer long crystal fragments compared to the untreated milk crystallization patterns (see figures 4.23 and 4.26).

These results are quite interesting as, like discussed above, PEF treatment did not lead to any significant changes in the structures of caseins and whey proteins of raw bovine milk, whereas heat treatment of milk caused whey protein denaturation and interaction with caseins (compare figures 4.1 on page 65 and 4.3 on page 68, respectively). Although the evidence of significant alterations for PEF and heat treated milk

is not as big as for skimming and HP treatment, this indicates that with the biocrystallization method, structural changes in raw milk can be detected that go beyond protein denaturation. Like discussed earlier, the inactivation of enzymes or micro-organisms or other undetected structural changes like nutrient losses could potentially play a role.

To sum up, with structure analysis it was shown that PEF and heat treatment both had similar effects on the pattern formation in the biocrystallograms: Less wide and fewer long crystal fragments were observed. This is interesting as unlike heat treatment, PEF treatment does not cause whey protein denaturation. It can be surmised that the inactivation of enzymes and micro-organisms also plays a role in pattern formation.

4.3 Suitability of picture forming methods to detect structural changes in milk

In the following it will be discussed whether each of the PFMs was able to detect the structural changes in milk caused by different types of processing and detected by standard analytical methods, above all HPLC. Furthermore, it is examined, whether other changes in the patterns of the pictures occurred that were not discovered by the standard analytical methods.

4.3.1 The informative value gained with the Steigbild method

Generally speaking, the results propose that the structural changes caused by different treatments are visible in the morphological characteristics of the Steigbild images. The results of the visual evaluation for the three treatments heat, HP and PEF are in line with the result obtained via HPLC. Hereby, interactions between casein (sub)-micelles and whey proteins as observed for the HP treated samples were visible in the form of rising inhibitions in the bowl zone and and and increased number of thinner flags. Probably, the rising inhibitions were caused by the formation of aggregates after contact with the salt solutions.

Whey protein denaturation as observed for the intense heat treatment (85 °C, 5 min) was shown in an even higher number of thin flags compared to HP treated milk. This means, the intact casein micelles of the heat treated milk covered with denatured whey proteins probably blocked the filter paper due to their bigger size which caused a local flow delay. Subsequently boundary lines between two adjacent zones of flow were formed which probably caused the occurrence of a higher number of flags.

However, with the Steigbild method it was not possible to distinguish between untreated and PEF treated milk samples as no clear and repeatable differences occurred between the images of the PEF treated and untreated milk. This suggests that the

state of proteins probably plays a major role in the pattern formation of the Steigbild images as effects were mainly observed for heat- and HP treatment but not for PEF treatment, where the proteins kept their native state.

Interestingly, next to the influence of proteins, also fat was observed to have an effect on the Steigbild images. The milk fat is probably directly linked with the occurrence of the corona, the whitish edge below the bowls as it was absent in the Steigbild images of the skimmed samples. This means that the Steigbild method is capable of showing structural changes in milk next to protein denaturation.

Yet, it must be noted that the reproducibility was very poor, which counts for all treatments. This can be linked to several factors. For example, the formation of reduction spots differed between the experiments. No link between the protein and fat contents of the samples and the appearance of reduction spots could be detected. It might be that the freshness of the salt solutions ($AgNO_3$ and $FeSO_4$) plays a role. Also, slight differences in climate or light during development or the temperature of the solutions are possible impacts.

It must be noted that the experimental setup was not ideal. A climate chamber as used for example in Wohlers (2011) would have serve better as the climate could have been more stable. In a bigger room where the experiments were conducted on a freely standing table accessible from all sides and with the option of dimmed light, also the rising of the different salt solutions could be monitored better. Mistakes like the rising of $AgNO_3$ above the 1 cm mark and maybe the occurrence of reduction spots could have been avoided in that way. Furthermore, monitoring and controlling of the sample temperature at the beginning of the first rising phase appears to be necessary as flow behaviour is mainly influenced by viscosity which changed with temperature.

One can conclude that the approach of Wohlers (2011) might be more feasible for the Steigbild method than the one used in this thesis and should be used for further experiments. However, her approach needs more equipment and a separate chamber which might not be available in all (research) institutions.

The online image analysis was a good tool for the visual evaluation of the images. However, some slight changes observed by the author were not detected by the test subjects. It might be interesting to repeat the experiments with a trained panel. Probably, very subtle variations in the images as for the low-intensity heat treated samples with the would have been detected much better, compare Wohlers (2011).

4.3.2 The informative value gained with circular chromatography

The results of the carried out descriptive test show clearly that circular chromatography as conducted in this thesis was not very suitable to detect structural changes in milk. The differences between the repetitions were bigger than the differences among the differently treated samples. In fact, no clear morphological changes were observed

regarding the changes caused by the milk treatments. This means, circular chromatography as conducted in this thesis is not sensitive enough to detect structural changes in proteins and the general composition (skimming). It has to be mentioned that there exist several different methodological approaches to this method. Therefore it might be that structural changes could be detectable with a different approach. For example, Balzer-Graf and Gallmann (2000) found differences in the middle and outer zone of HP treated milk. Also, Beckmann et al. (1993) detected differences in the chromatograms of milk that was heated up to 85 °C for 8-10 s. These findings could not be confirmed in the experiments of this thesis and therefore the chosen methodological approach might have been unsuitable.

Generally, the method is very prone to light conditions and other outer influences. It was observed that colour development was not completed after three hours as the chromatograms darkened even during storage. After approximately one week of storage between paper in a dark place, no change in colour was observed anymore. During that time, mostly the spikes in the outer zone darkened in colour proving that colour development is not necessarily only linked to light exposure. Therefore, the chromatograms would have had to be developed for one week before evaluation which is a rather long time and thus circular chromatography is probably not a practical tool for food quality determination.

However, the method shows some other potential: the observed positive correlation between the area of lowest optical density around the center of the chromatogram and total protein content might be a suitable tool to indicate the protein content of milk samples next to standard analytical methods. On the other hand, precision might be a problem and so the methodology should be improved. Standardization is needed in any case.

4.3.3 The informative value gained with biocrystallization

As was evident from the results obtained with HP treated samples (600 MPa), casein and whey protein denaturation and their interactions had a clear effect on the crystal structure. Mainly, shorter needles were observed plus the occurrence of small dots that can be linked to the occurrence of flocculation after adding the $CuCl_2$-solution to the diluted milk. Several reasons are possible for this phenomenon which did not occur on all experimental days: cold denaturation during transportation or prolonged agitation are some of them. The changes in the biocrystallization patterns were detected with both visual evaluation and structure analysis.

The effect of heat treatment (85 °C) on the crystallization patterns proved to be less significant than for the HP treatment. The differences were only detected by structure analysis and by the author but not by the test subjects of the online visual evaluation. Needles were slightly shorter and broader, which is interesting, as no effect

on the needle width was observed for the HP treated samples, where whey protein denaturation and its interaction with caseins were similar.

Interestingly, not solely protein denaturation of heat and HP, but also other changes in the milk composition caused changes in the crystal structure. This was especially the case for the alteration of the fat content (skimming). Hereby, the formation of a haze was observed with some clear transparent spots appearing behind the crystal structure on the edge of the plates. Probably, the clear transparent spots are linked with residual fat. Self organization processes like in Bénard cells might have taken place and transported the fat to the edge of the plate.

Furthermore, the influence of the PEF treatment was detected with structure analysis, although the differences were not as significant as they proved to be for skimming and HP treatment and were not detected via the online image analysis. It remains unclear, by what these subtle effects were caused, as no protein denaturation took place. Literature mentions the inactivation of micro-organisms and enzymes as main impacts of PEF, next to other potentially unknown side effects. In can only be guessed at this point, how big the impact of these subtle changes are on the crystallization pattern.

Next to the visual evaluation, the structure analysis tool proved to be a suitable tool to detect even fine changes in the crystallization patterns caused by different treatments of raw bovine milk. Texture analysis failed to detect any differences and so its suitability for milk samples can be questioned. In most cases, the results of the structure analysis were in line with the outcomes of the online image analysis and the findings of the author concerning the morphological aspects. Unlike the other two PFMs, the biocrystallization method consistently produced reproducible results, especially in online visual analysis. Results of the structure analysis were not as reproducible, probably because more subtle differences between the samples were detected that were not visible with the human eye or the statistical power was too low. This could have been prevented by analyzing more plates. Yet, the number of plates per treatment and day with twelve plates was already quite high. It can only be guessed whether an additional day repetition would have solved the problem. The need of a large amount of plates is definitely a weak point of the biocrystallization method.

Although all these unknown side effects occurred the biocrystallization method can be seen as the most promising of all PFMs as it shows a good reproducibility and a relatively high sensitivity for treatment induced changes in milk samples. Further research is needed to discover the exact influencing factors that lead to the formation of (altered) patterns.

5 Conclusions and outlook

The aim of this thesis was to find out, whether the picture forming methods Steigbild, circular chromatography and biocrystallization are sensitive to modifications of proteins in milk and whether other structural changes that occur during processing can be detected as well. For this purpose, these methods were compared to standard analytical methods such as HPLC for the detection of changes caused by four thermal and non-thermal treatments of raw bovine milk.

In summary it can be said that the result of the Steigbild method were in line with the result of HPLC. Whey protein denaturation and subsequent interaction with the surface of the casein micelles led to and increased number of flags. Additionally, the disruption of the casein micelles as found for HP treated milk showed itself in the occurrence of flow inhibitions in the bowl zone. Probably, contact with the salt solutions led to aggregation and therefore increased blocking of the filter pores. PEF treatment had no effect on the pattern formation of the Steigbild images which is in line with HPLC results where no modification of milk proteins was observed. Interestingly, skimming caused the corona under the bowls to disappear and therefore the appearance of the corona can be directly linked to the milk fat. Reproducibility was generally poor for all conducted experiments. This indicates that the approach of Wohlers (2011) who used a climate controlled chamber is more suitable for the Steigbild method. With the online image analysis, a new suitable tool for objective image analysis was provided.

Circular chromatography was unable to detect any differences caused by the various thermal and non-thermal treatments. Furthermore, poor reproducibility was a great problem of the method which was caused by differences in colours between the sample repetitions. As the method is not standardized, the methodology chosen in this thesis could have been not suitable. Yet, circular chromatography showed a potential for determination of the protein-content of milk, as the whitish area around the center was linked with protein-concentration, which was already suggested by Knorr (1982) in the case of protein solutions and collard plants. Therefore, circular chromatography offers a low-cost alternative for protein content determination.

Of all three PFMs, biocrystallization was the only one to show reproducible results. With the tool of structure analysis, fine differences in the needle structure of the biocrystallograms were detected. It was shown that all milk treatments caused changes in length of the crystal needles and for all treatments besides HP, changes in the width of needles was observed.

For the HP treated samples, flocculation occurred during sample preparation which might be linked to prolonged agitation and the formation of larger aggregates

out of casein (sub-)micelles and denatured whey proteins which were not present after HP treatment. Next to that, shorter needles were observed for HP treated compared to untreated samples. Heat treatment caused the occurrence of thinner next to shorter needles. This is interesting, as mechanisms of whey protein denaturation are similar for heat and HP treated milk. It can be surmised that the disruption of caseins and the formation of flocculation outweigh the influence of whey protein denaturation on needle width. It has to be noted, though that the results of heat treatment were not as significant as for HP treatment.

Skimming of milk had a very interesting effect on the appearance of the crystallograms. A haze occurred behind the crystal structure. On some plates, clear transparent spots were visible in the outer zone which might be linked to residual milk fat and the occurrence of Bénard-like cells in which the creamed residual fat was carried to the edge of the plate. Furthermore, thicker and thinner needles were found. It must be noted that a small influence of PEF on pattern formation was observed. Hereby, the changes resembled the ones found for the heat treated samples. This is interesting as no modification of milk proteins was observed with HPLC. It can be guessed that the denaturation of enzymes and micro-organisms also plays a role in pattern formation.

A deficit in statistical power was observed for structure analysis of PEF and heat treated samples. This suggests that a larger number of plates would have been needed to obtain significant results. As already a high number of samples was used for each analysis, this represents a weak point of the biocrystallization method. Yet, the results clearly show that next to protein denaturation also other structural changes in milk are detectable with the biocrystallization method.

The different approach of the PFMs ('top-down') compared to standard analytical methods ('bottom-up') turns PFMs into a valuable addition next to standard analytical methods. Whereas detailed information about single compounds or fractions can be gained by standard analytical methods, PFMs offer an integrated view on the changes caused by different thermal and non-thermal treatments of raw bovine milk. Next to that, the methods offer low cost analysis. Due to their simple methodology, all PFMs are relatively cheap compared to most standard analytical methods.

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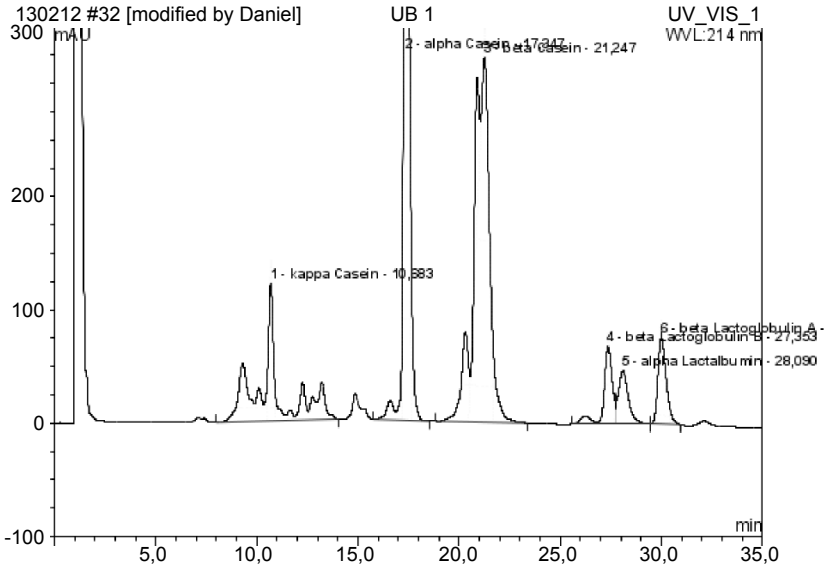
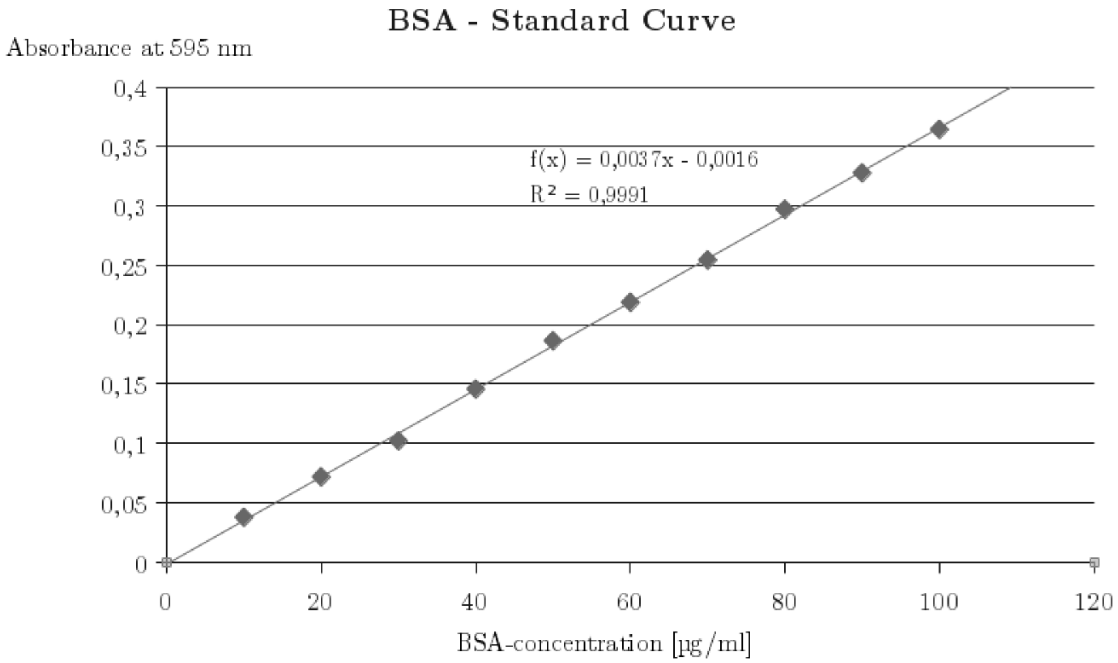
APPENDICES

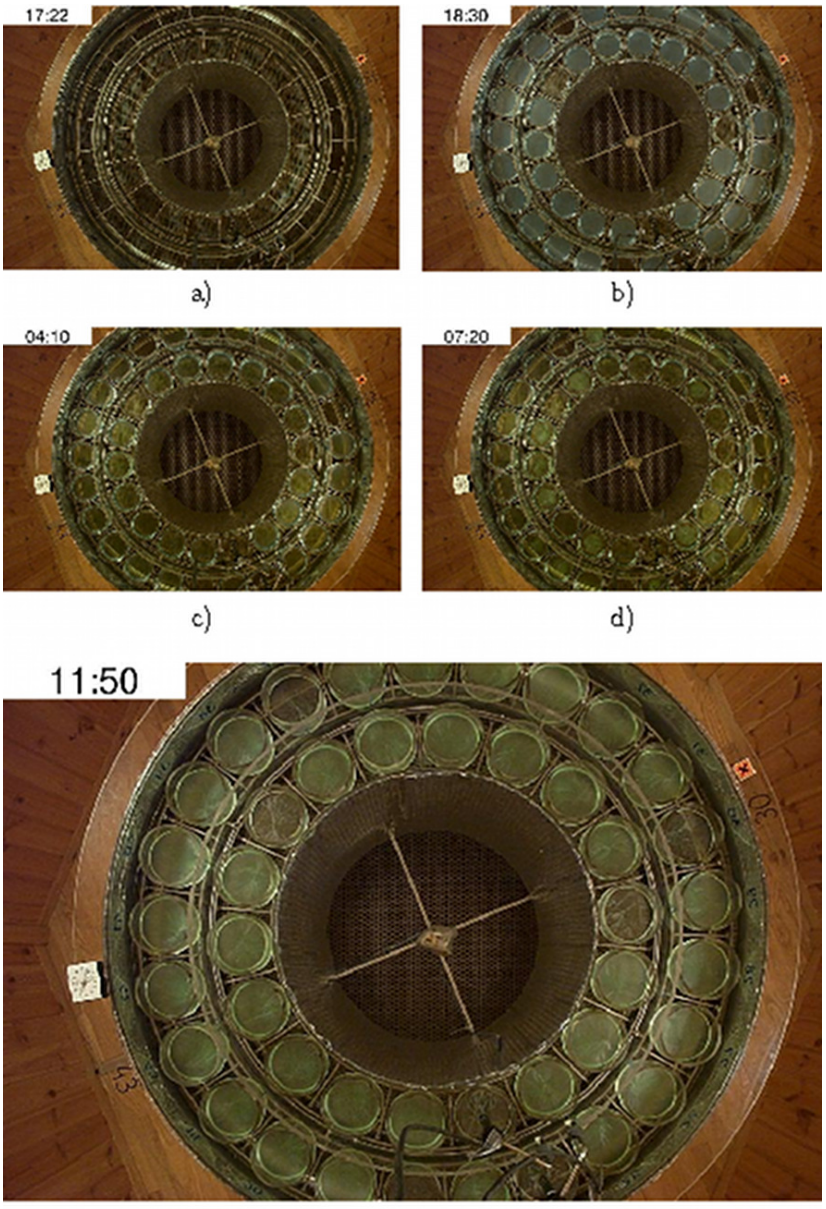
A HPLC-Chromatogram of a raw untreated milk sample



B BSA standard curve

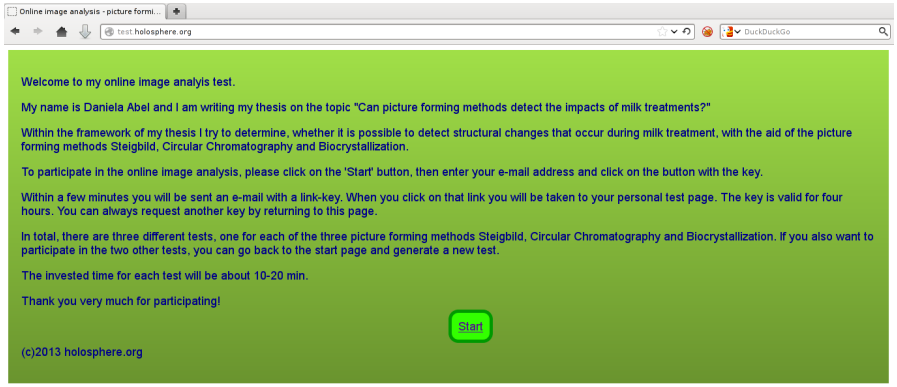


C Monitoring of nucleation process

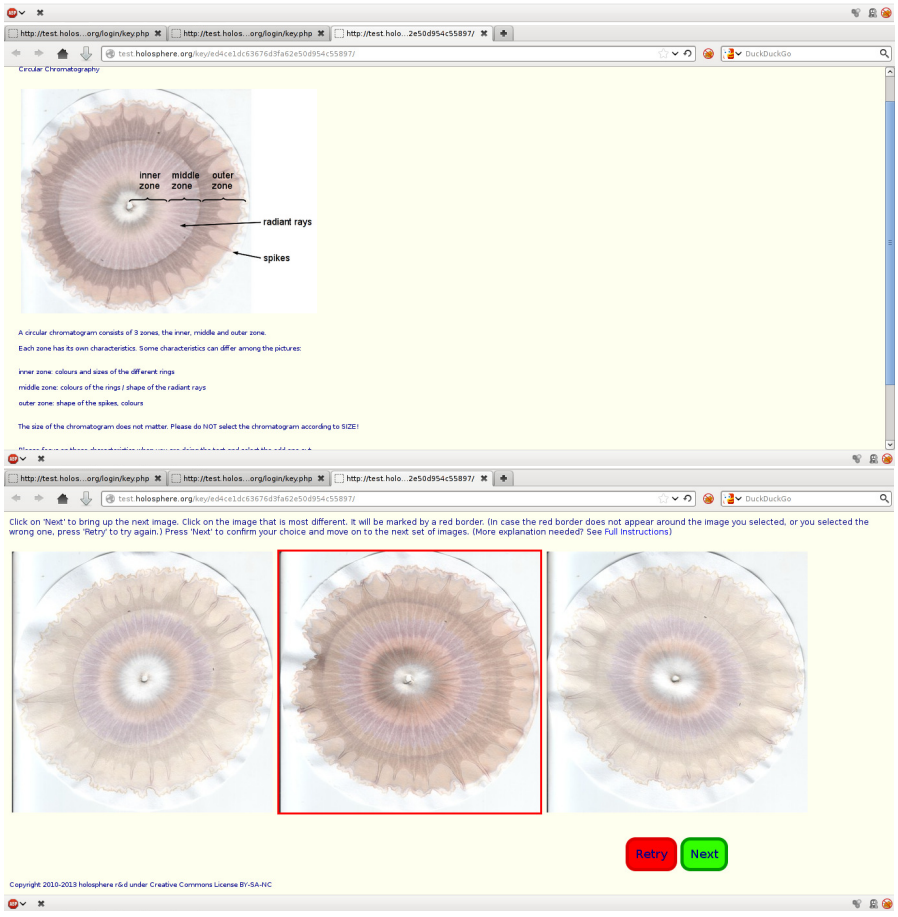


Monitoring of the crystallization process in the biocrystallization experiments over time: a) inner chamber with two rings, b) crystallization dishes with pipetted samples in chamber, c) clearing of the samples before nucleation, d) starting of nucleation processes, d) crystallization process finished.

D Screen-shot of the online image analysis homepage



Introduction text



Explanation

Test-triad

E Protein and fat contents of the milk samples sorted by date