Master Thesis

Influence of Ultra-High-Pressure-Homogenisation on the emulsifying properties of egg yolk, applied to model – emulsion - system.

Alexander Meißner

14.01.2013

Supervisor 1: Prof. Dr.-Ing. Peter Meurer (University of Applied Sciences Neubrandenburg)

Supervisor 2: Dr.-Ing. Tomas Bolumar (German Institute of Food Technologies)

Supervisor 3: Dr.-Ing. Waldemar Buxmann (German Institute of Food Technologies)

Abstract

The novel method ultra-high pressure homogenisation (UHPH) allows homogenising fluids with up to 350 MPa, which corresponds to 30 times higher pressure in comparison with the conventional homogenisation process. The aim of this master thesis is to determine the effect that UHPH-processing has to the conventional homogenised and pasteurised egg yolk and the thermostable egg yolk (enzymatically modified by phospholipase A$_2$) with respect to the emulsification and stabilization ability in model-emulsions. The main focus of the investigations of this work was to study the structural changes taking place in the egg yolk dilution (1:1; 0.17 M sodium chloride) by ultra-high pressure homogenization and its effect on the microstructure of the emulsion (O/W; 50/50 with protein content of 2%) with respect to emulsifying, stabilizing and rheological behaviour.

The enzyme modification of egg yolk by phospholipase A$_2$ (PLA$_2$) increases the viscosity and stability of the model-emulsions compared to conventional egg yolk. Phospholipase A$_2$ modification protected the structure of egg yolk against the high forces and temperatures generated during the UHPH-processing. The UHPH-processing induces protein modifications by unfolding the protein structure which results in an increased crosslinking of the proteins, lyso- and phospholipids and finally results in a denser network. This network and the improved stabilization of the interfacial films of oil droplets generates in the emulsion a higher viscosity and stability. The UHPH-processing of conventional egg yolk had decreased the viscosity in emulsion, regardless of the pressure level of homogenization, and shows no improvement in the stability. The homogenization pressure of 225 MPa was incorporated as the optimal processing conditions which generated the best balance between viscosity and stability in emulsion. The pressure range of 100 to 150 MPa enabled the egg yolk to generate high viscosities and lower stability in emulsion and vice-versa to the pressure range of 300 to 350 MPa.
Acknowledgements

I would like to thank the German Institute of Food Technologies and Professor Stefan Töpfl for providing me with the highly interesting and complex theme. I would also like to thank Professor Peter Meurer for taking supervision of my work. I would like to express my deep gratitude to my supervisors Dr. Tomas Bolumar and Dr. Waldemar Buxmann, for the valuable support and guidance they have given me throughout the whole project.
# Table of Contents

ABSTRACT ........................................................................................................................................ I
ACKNOWLEDGEMENTS ............................................................................................................... II
LIST OF TABLES ................................................................................................................... V
LIST OF FIGURES ................................................................................................................ VI

1. INTRODUCTION AND OBJECTIVE TARGET ........................................................................ 1
2. THEORETICAL PRINCIPLES .................................................................................................. 3

## 2.1 Emulsions
2.1.1 Definition .......................................................................................................................... 3
2.1.2 Properties of Oil in Water Emulsions ................................................................................. 3
2.1.3 Destabilising Mechanisms ............................................................................................... 5
2.1.4 Emulsifiers ....................................................................................................................... 8
2.1.5 Emulsifying Properties of Proteins ............................................................................... 10
2.1.6 Stabilisers ....................................................................................................................... 11

## 2.2 Egg Yolk
2.2.1 Composition of the Egg Yolk ......................................................................................... 12
2.2.2 Proteins of Egg Yolk ....................................................................................................... 13
2.2.3 Lipids ............................................................................................................................... 15
2.2.4 Emulsifying Properties of Egg Yolk ............................................................................. 16
2.2.5 Thermal Behaviour of Egg Yolk Proteins ..................................................................... 17

## 2.3 Thermostable (enzyme modified) Egg Yolk
2.3.1 Enzymatic Modification by Phospholipase .................................................................. 18
2.3.2 Hydrolysis Mechanism of Phospholipase A2 (PLA2) .................................................... 19
2.3.3 Changes in the Properties of Egg Yolk by the Hydrolysis with Phospholipase A2 ...... 19

## 2.4 Homogenisation Processes in Industry
2.4.1 Homogenisation Systems ............................................................................................... 20
2.4.2 Ultra-High Pressure Homogeniser “FPG11300 Hygenic Homogeniser” ...................... 24

## 2.5 State of Science - influence of high pressure homogenization on the properties of emulsions and their components

3. MATERIALS AND METHODS ............................................................................................. 29

## 3.1 Material

## 3.2 Methods
3.2.1 Ultra-High Pressure Homogenization of Egg Yolk......................................................... 31
3.2.2 Method for Producing O/W-Emulsions ........................................................................ 32

## 3.3 Analytical Methods for Characterization of Egg Yolk
3.3.1 Dynamic Interfacial Tension ......................................................................................... 35
3.3.2 Scanning Electron Microscopy (SEM) ......................................................................... 35

## 3.4 Analytical Methods for Characterization of O/W Emulsions
3.4.1 Rheology ......................................................................................................................... 36
3.4.2 Stability Tests .................................................................................................................. 37
IV

3.4.3 Droplet Size Distribution and Surface to Volume Ratio ..................................................... 38
3.4.4 Protein-Interface-Occupancy ............................................................................................. 39
3.4.4.1 Separation of the Continuous and Disperse Phases ......................................................... 39
3.4.4.2 Determination of Protein Content of Dispersed Phase ...................................................... 39
3.4.4.3 Calculation of Protein-Interfaces-Occupancy of Emulsions ............................................... 40
3.4.5 Extractable Fat Fraction (EFF) .......................................................................................... 40
3.4.5.1 Determination of total Fat Content of Emulsion ................................................................. 41
3.4.6 Confocal Laser Scanning Microscope (CLSM) .................................................................. 41

4. RESULTS AND DISCUSSIONS ................................................................................... 43

4.1 Pilot-Tests 1....................................................................................................................... 43
4.1.1 Results of Pilot-Tests 1 ................................................................................................. ..... 43
4.1.1.1 Temperature Development of the Premixes ...................................................................... 43
4.1.1.3 Macroscopic Stability of the Emulsions ............................................................................. 46
4.1.2 Discussion of Pilot-Tests 1................................................................................................. 49

4.2 Pilot-Tests 2....................................................................................................................... 50
4.2.1 Results of Pilot-Tests 2 ................................................................................................. ..... 51
4.2.1.1 Rheological Characteristics of Emulsions .......................................................................... 51
4.2.1.2 Droplet Size Distribution and Surface to Volume Ratio ..................................................... 54
4.2.1.3 Macroscopic Stability of Emulsions ................................................................................... 59
4.2.2 Discussion of Pilot-Tests 2............................................................................................... .. 60

4.3 Main-Tests......................................................................................................................... 62
4.3.1 Results of Egg Yolk............................................................................................................ 63
4.3.1.1 Analysis of Dynamic Interfacial Tension ............................................................................ 63
4.3.1.2 Investigations of Egg Yolk Structure by SEM-Images ....................................................... 64
4.3.2 Results of Emulsion ......................................................................................................... 65
4.3.2.1 Rheological Characteristics of Emulsions .......................................................................... 65
4.3.2.2 Droplet Size Distribution and Surface to Volume Ratio ..................................................... 66
4.3.2.3 Macroscopic Stability of Emulsions ................................................................................... 67
4.3.2.4 Protein-Interfaces-Occupancy of Emulsions ...................................................................... 69
4.3.2.5 Extractable Fat Fraction (EFF) of Emulsions ..................................................................... 69
4.3.2.6 Investigations of Emulsion by CLSM-Images .................................................................... 70
4.3.3 Discussion of Main-Tests ................................................................................................. .. 74
4.3.3.1 Influence of Ultra-High Pressure Homogenization (UHPH) Processing in Egg Yolk......... 74
4.3.3.2 Influence of UHPH-Processing of Egg Yolk on the Microstructure and the Interfacial Film of the Emulsion. ................................................................................................................................. 75
4.3.3.3 Influence of UHPH-Processing on the Emulsifying Behaviour of Egg yolk in an Emulsion................................................................................................................................. 76
4.3.3.4 Influence of UHPH-Processing on the Stabilization Abilities of Egg Yolk in an Emulsion . 77
4.3.3.5 Influence of UHPH-Processing on the Rheological Characteristics of Egg Yolk in an Emulsion................................................................................................................................. 78

5. SUMMARY AND CONCLUSIONS ............................................................................... 79

6. FUTURE RECOMMENDATION ................................................................................... 81

7. REFERENCES ............................................................................................................. 83

8. APPENDIX ................................................................................................................... 91

9. SELBSTSTÄNDIGKEITSERKLÄRUNG ...................................................................... 93
List of Tables

Table 1. Pilot-Tests 1: Composition of the O/W emulsions of unmodified egg yolk as well as modified PLA₂ egg yolk (O/W; 50/50 emulsion with a protein content of 1.25% in the aqueous phase) .............................................................................33

Table 2. Pilot-Tests 2: Composition of the O/W emulsions of PLA₂-modified yolk (O/W; 50/50, 35/65 or 10/90) emulsion with a protein content of 1.25% or 1.625 in the aqueous phase ...........................................................................34

Table 3. Main-Tests: Composition of the O/W emulsions of PLA₂-modified egg yolk (O/W; 50/50 emulsion with a protein content of 2% in the aqueous phase). ..........34

Table 4. Flow Behaviour Index by Ostwald de-Waele of all tested O/W model emulsion, produced with the Premixes of PLA₂-modified egg yolk which was treated by HPH at different pressures, after 1 and 10 days at 7 °C. .................................................91

Table 5. Effect of oil concentration (10, 35 and 50%) and HPH (200, 225, 250, 300 MPa) treated protein content (1.25 and 1.625%) in O/W emulsions on their sauter mean diameter, dispersion index (SPAN) and specific surface area, after 1 and 10 days at 7 °C. ........................................................92
List of Figures

Figure 1. Construction of O/W- and W/O emulsions [Schuchmann and Danner, 2004]........... 3
Figure 2. Schematic illustrations of the types of instabilities in emulsions [Capek, 2004] ...... 6
Figure 3. Schematic representation of a phospholipid (lecithin) to the bounding surface area oil / aqueous phase. ................................................................. 8
Figure 4. Schematic representation of the adsorption flexible proteins at the oil / water bounding surface [Myers, 1991].........................................................10
Figure 5. Composition of the egg yolk (in dry matter) [Acker und Ternes et al., 1994] .........12
Figure 6. Micelle structure of LDL: A: Apo-LDL, B: Phospholipids C: Triacylglycerides (modified through Buxmann [2009]; [Kamat et. al., 1972; Anton et. al,. 2003; Ternes, 2008]). .....................................................................................................14
Figure 7. Cleavage sites of phospholipases on the phospholipid molecule [Markert 2004]. ..................................................................................................................................................18
Figure 8. Example of a discontinuous working rotor-stator system [McClements, 2005]. ......20
Figure 9. Composition of a colloid mill [McClements, 2005]. .................................................21
Figure 10. Schematic of a discontinuous working ultrasonic system [Schuchmann and Danner, 2004]. ...................................................................................................................22
Figure 11. Schematic principle of membrane emulsification [Leal-Calderon et. al., 2007]. ...........................................................................................................................................22
Figure 12. Different types of high-pressure homogenisation valves [Stang et. al., 2001]......23
Figure 13. Summary overview of various methods for emulsification [Schultz et. al., 2004]. ...................................................................................................................24
Figure 14. Simple scheme of the structure of “FPG11300 Hygenic Homogeniser” [Handbook FPG11300, 2011]. ........................................................................................................25
Figure 15. Schematic representation of the effect of ultra-high pressure on the product temperature [Donsi et. al., 2009]. ..........................................................................................26
Figure 16. Schematic representation of the ultra-high pressure needle valve system (modified after Floury et al [2004a and 2004b]).........................................................27
Figure 17. An emulsion containing flocculated droplets exhibits shear-thinning behaviour because the flocs are progressively aligned, deformed and disrupted in the shear field [McClements, 2005]. ..........................................................................................................................28
Figure 18. Production steps for manufacturing of Premix..........................................................32
Figure 19. Production steps for preparation of O/W Emulsions .............................................33
Figure 20. Effect of homogenizing pressure on the temperature of the Premixes (unmodified and PLA2-modified egg yolk) at the exit of the first valve. Initial temperature of the Premix: 5 ° C. .................................................................43
Figure 21: Flow Consistency Index by Ostwald de-Waele of the O/W (50/50) model-emulsions with a protein content of 1.25%, produced with the Premixes of unmodified and PLA2-modified egg yolk which was treated by HPH at different pressures, after 1 day at 7 ° C .................................................................44
Figure 22: Flow Behaviour Index by Ostwald de-Waele of the O/W (50/50) model-emulsions with a protein content of 1.25 %, produced with the Premixes of unmodified and PLA2-modified egg yolk which was treated by HPH at different pressures, after 1 day at 7 ° C .................................................................45
Figure 23. Stability test of the O/W (50/50) model-emulsions with a protein content of 1.25 %, prepared with the Premixes of unmodified and modified PLA2 egg yolk and treated by HPH at different pressures, after 1 day at 25 ° C. .......... 46
Figure 24. Stability test of the O/W (50/50) model-emulsions with a protein content of 1.25 %, prepared with the Premixes of unmodified and modified PLA\textsubscript{2} egg yolk and treated by HPH at different pressures, after 3 days at 25 °C. ..................47

Figure 25. Stability test of the O/W (50/50) model-emulsions with a protein content of 1.25%, prepared with the Premixes of unmodified and modified PLA\textsubscript{2} egg yolk and treated by HPH at different pressures, after 7 days at 25 °C. ..................48

Figure 26. Flow Consistency Index by Ostwald de-Waele of O/W (10/90) model-emulsion with different protein content (1.25% and 1.625%), produced with the Premixes of PLA\textsubscript{2}-modified egg yolk which was treated by HPH at different pressures, after 1 and 10 days at 7 °C. ..................................................51

Figure 27. Flow Consistency Index by Ostwald de-Waele of O/W (35/65) model-emulsion with different protein content (1.25% and 1.625%), produced with the Premixes of PLA\textsubscript{2}-modified egg yolk which was treated by HPH at different pressures, after 1 and 10 days at 7 °C. ..................................................52

Figure 28. Flow Consistency Index by Ostwald de-Waele of O/W (50/50) model-emulsion with different protein content (1.25% and 1.625%), produced with the Premixes of PLA\textsubscript{2}-modified egg yolk which was treated by HPH at different pressures, after 1 and 10 days at 7 °C. ..................................................53

Figure 29. Effect of oil concentration (10, 35 and 50%) and HPH level (200, 225, 250, 300 MPa) on the sauter mean diameter of O/W emulsions with protein content (1.25 and 1.625 %), after 1 day at 7 °C. ..................................................54

Figure 30. Effect of oil concentration (10, 35 and 50%) and HPH level (200, 225, 250, 300 MPa) on the dispersion index (SPAN) of O/W emulsions with protein content (1.25 and 1.625 %), after 1 day at 7 °C. ..................................................55

Figure 31. Effect of oil concentration (10, 35 and 50%) and HPH level (200, 225, 250, 300 MPa) on the specific surface area of O/W emulsions with protein content (1.25 and 1.625 %), after 1 day at 7 °C. ..................................................56

Figure 32. Effect of oil concentration (10, 35 and 50%) and protein content (1.25 and 1.625 %) on the oil droplet size distribution of O/W emulsions, after 1 day at 7 °C. ..................................................57

Figure 33. Effect of HPH treatment of protein content (1.25 %) on the oil droplet size distribution of O/W (50/50) emulsions, after 1 day at 7 °C. ..................................................57

Figure 34. Effect of HPH treatment of protein content (1.25 %) in O/W (50/50) on the oil droplet size distribution of O/W (50/50) emulsions, after 10 days at 7 °C. ..................................................58

Figure 35. Effect of oil concentration (10, 35 and 50%) and HPH (200, 225, 250, 300 MPa) treated protein content (1.25 and 1.625 %) in O/W emulsions on its stability, after 14 days at 7 °C. ..................................................59

Figure 36. Dynamic Interfacial Tension between Miglyol and diluted Premix (1/100 w/w; 0.08% protein content) of PLA\textsubscript{2}-modified or non-modified and treated or untreated egg yolk as a function of the droplet formation time. ..................63

Figure 37. SEM-images of Premixes (diluted egg yolk; 50/50 w/w). a: unmodified and untreated egg yolk; b: PLA\textsubscript{2}-modified and untreated egg yolk; c: PLA\textsubscript{2}-modified and 225 MPa treated egg yolk; d: PLA\textsubscript{2}-modified and 300 MPa treated egg yolk (2% protein content); at 2500 magnification. ..................64

Figure 38. Flow consistency index [K] and Flow Behaviour Index [n] by Ostwald de-Waele of the O/W (50/50) emulsions with PLA\textsubscript{2}-modified or unmodified and treated or untreated egg yolk (2% protein content). ..................65
Figure 39. Oil droplet size distributions and specific surface area of the O/W (50/50) emulsions with PLA$_2$-modified or unmodified and treated or untreated egg yolk (2% protein content). .................................................................66

Figure 40. Stability of the O/W (50/50) emulsions with different types of egg yolk (2% protein content). UM: unmodified and untreated egg yolk; 0: PLA$_2$-modified and untreated egg yolk; 225: PLA$_2$-modified and 225 MPa treated egg yolk; 300: PLA$_2$-modified and 300 MPa treated egg yolk; at day 1 / 3 / 7 / 10 after production.................................................................67

Figure 41. Stability of the O/W (50/50) emulsions with different types of egg yolk (2% protein content). UM: unmodified and untreated egg yolk; 0: PLA$_2$-modified and untreated egg yolk; 225: PLA$_2$-modified and 225 MPa treated egg yolk; 300: PLA$_2$-modified and 300 MPa treated egg yolk; at day 14 after production.................................................................68

Figure 42. Protein-Interfaces-Occupancy of the O/W (50/50) emulsions with PLA$_2$-modified or unmodified and treated or untreated egg yolk (2% protein content). .................................................................69

Figure 43. Extractable Fat Fraction (EFF) of the O/W (50/50) emulsions with PLA$_2$-modified or unmodified and treated or untreated egg yolk (2% protein content). .................................................................70

Figure 44. CLSM images of the oil droplet interface of the O/W (50/50) emulsions with unmodified and untreated egg yolk (2% protein content) at different magnifications (left pic. 10x; right pic. 100x). ..............................................71

Figure 45. CLSM images of the oil droplet interface of the O/W (50/50) emulsions prepared with PLA$_2$-modified and untreated egg yolk (2% protein content) at different image magnifications (left. pic. 10x; right. pic. 100x). ........................72

Figure 46. CLSM images of the oil droplet interface of the O/W (50/50) emulsions prepared with PLA$_2$-modified and by 225 MPa treated egg yolk (2% protein content) at different image magnifications (left pic. 10x; right pic. 100x). ..............................72

Figure 47. CLSM images of the oil droplet interface of the O/W (50/50) emulsions prepared with PLA$_2$-modified and by 300 MPa treated egg yolk (2% protein content) at different image magnifications (left pic. 10x; right pic. 100x)........73
1. Introduction and Objective Target

The hen egg is one of the most versatile foods. Eggs are commonly used in many food industries as a raising agent or emulsifier (bakery, meat, noodles, ice cream, …). These ingredients are added in the manufacturing process as whole-egg, egg white or egg yolk and in different forms such as liquid, concentrated, dried, crystallized, frozen, deep frozen or fermented. The present work focuses on the emulsifying properties of egg yolk employed in oil in water emulsion (for instance: mayonnaise). The emulsification properties are based on the composition of egg yolk. On the one hand, there are the ingredients proteins and phospholipids and on the other hand, there are some specific structural elements such as Phosvitin of the Granule fraction, Low-Density-Lipoproteins of the Plasma fraction and others [Acker and Ternes et al., 1994].

The good emulsifying properties of egg yolk have been known for millennia. Nevertheless understanding what happens at the molecular level started three decades ago. This research was the result of the changes in the world of work and society. The workers/consumers needed fewer calories to work, but they did not give up their eating habits. The food industry recognizes the gap in the market and they produce low-fat products for over thirty years. The low-fat products contained a large number of stabilisers and other additives to get the same characteristics like the standard products. The consumers changed again their claims in the recent years. Nowadays they want product with natural ingredients and without additives, but without changing the properties (e.g. less fat). The latest manufacturing techniques (e.g. Ultra High Pressure Homogenisation) enable the food industry to optimise their products towards consumer needs.

The aim of this master thesis is to determine which effect has the novel method, Ultra High Pressure Homogenisation (UHPH), to the conventional homogenised and pasteurised egg yolk. In particular, the analysis of the functional properties of egg yolk as emulsifier and stabiliser and the comparison of these to untreated material. For this purpose egg yolk will be treated at different pressures. An emulsion model based on a mayonnaise recipe should serve as a vehicle to illustrate the differences.

The pilot plant allows homogenising fluids with up to 350 MPa, which corresponds to 30 times higher pressure in comparison with the conventional homogenisation process. The higher pressure causes stronger shear forces. Theoretically, proteins are unfolded at increasing shear forces and this leads to an enlargement of their protein network. Therefore it is important to clarify whether the structure of the egg yolk proteins are unfolded and if so, if this effect will increase the emulsifying and stabilising capacity of egg yolk due to the larger network of proteins and to which processing conditions this happens. Furthermore, a higher
pressure generates in addition to larger shear forces also increased frictional forces, wherein the released thermal energy increases, which the non-enzyme-modified egg yolk proteins denatured from 65 to 70 °C. For this reason, this work examines the influence of the pressure and temperature on the proteins of egg yolk and the consequent of changes in the emulsifying and stabilising capacity of the model-emulsion.

Based on the results of the previous work by Marco-Moles et al. [2012] who observed an unstable emulsion at a homogenization pressure of 250 MPa, and the well-known effect of HPH processing on the raising of the temperature of the product and the negative effect this has on the protein denaturation and thus in the functionality, in this work it is included conventional egg yolk and in addition thermostable egg yolk (modified by phospholipase A2) which could be more resistant to heat exposition.

The main objective of this study is to find the optimal type of egg yolk, recipe and processing conditions which enhances the emulsifying and stabilising properties of egg yolk and thus enable the reduction in the proportion of fat/oil in the emulsion.
2. Theoretical Principles
2.1 Emulsions

2.1.1 Definition

The definition of an emulsion by the “International Union of Pure and Applied Chemistry (IUPAC)” is as follows: “In an emulsion liquid droplets and/or liquid crystals are dispersed in a liquid. An emulsion is denoted by the symbol O/W if the continuous phase: is an aqueous solution and by W/O if the continuous phase is an organic liquid (an oil).” [IUPAC, 1972.]

![Figure 1. Construction of O/W- and W/O emulsions [Schuchmann and Danner, 2004].](image)

An emulsion, in simple terms, is a mixture of an aqueous phase and a fat containing phase. The emulsion type depends on the proportion of two different phases (Figure 1) in a system. In water in oil (w/o) emulsions systems (e.g. butter), water is dispersed in a continuous oil phase, whereas in oil in water (O/W) emulsion (e.g. mayonnaise), oil is the dispersed phase in a water phase [Helmerich, 2004; Belitz et al., 2005: Dis Waldemar]. Butter and margarines are W/O emulsions, while mayonnaise, sauces, milk and salad dressings belong to O/W emulsions.

Double emulsions (O/WO or W/OW) arise by re-emulsifying the emulsion. Multiple emulsions are also possible [Tscheuchner, 1996].

2.1.2 Properties of Oil in Water Emulsions

Basic elements of the emulsion are the internal/disperse phase and the external/continuous phase. The internal phase consists of fine droplets into the external phase [Tscheuchner, 1996]. These basic components are intrinsically immiscible. An emulsion can be produced by applying sufficient energy input (to overcome the surface tension see below) and/or using adequate amount of emulsifiers (to lower the interfacial tension between water and oil) [Ternes et. al., 1994; Helmerich, 2004].
Emulsions are a temporary phenomenon that means they are stable for a few hours up to several years. The stability time of emulsions depends on composition (volume fraction and ingredients), droplet size of disperse phase or energy input. Stabilizers and emulsifiers constitute the main factors for stability [Tscheuchner, 1996; Ternes, 2008].

**Volume Fraction**

Volume fraction of dispersed phase indicates the percentage of oil in an emulsion. This oil content determines the consistency of an emulsion and it is just as important for their characterization [Tesch, 2002].

**Oil Droplet Size**

Qualitative statements about an emulsion can be made by determination of oil droplet size. Oil droplet size determines visual characteristics of emulsion, such as colour and gloss [Friberg, 1997]. Emulsions have a milky appearance if the oil droplets are larger than 0.1 μm and nanoemulsions appear clear because their oil droplets are smaller than 0.1 μm [Kunz, 1993]. Furthermore oil droplet size, texture and stability have influence on the properties of emulsions. High stability of an emulsion is generally brought into relation with a fine and uniform structure [Harrison and Cunningham, 1985]. In reality, in food emulsions, mostly bimodal or polymodal oil droplet size distribution exists, uniform structure (monomodal) accrue rather seldom. For this reason, it is not enough to describe an emulsion using only reference to the average values of the oil droplet size (i.e. d 0.1; d 0.5; d 0.9), but also with respect to consideration of the droplet size distribution curve.

**Interfacial Tension**

The interface is the area at the transition between two phases. The interfacial tension arises from the inward force at the interface and it is established by the intermolecular interactions [Bade, 2005]. The thermodynamic properties change abruptly at the interface [Pohl, 2005]. The system of interface is always striving to achieve an energetically favourable state. This means that each increase of the interface brings the molecules of the dispersed phase in an energetically unfavourable state, which is counteracted by a minimization of the interface [Pohl, 2005]. Therefore needs to be invested for each increase in the interface energy in the form of mechanical work.
Viscosity

Several factors affect the viscosity of an emulsion. The greatest influence of the volume fraction has the droplet size and the viscosity of the continuous phase. In turn, the viscosity of the continuous phase is depending on the dissolved substances (emulsifiers and / or stabilisers) in it and the droplet size distribution [Gerhards, 2005]. In addition, the viscosity can be influenced by substances on the interfacial film of the droplet surface, which interact with the continuous phase and may finally lead to viscosity increase.

The specific interface area determines the proportion of continuous phase which may be immobilised by the disperse phase.

The effect of emulsifiers on the viscosity of emulsions is dependent on the type of emulsifiers. The viscosity of the emulsion is mainly influenced by the average particle diameter in the use of low molecular emulsifiers. However, in emulsions with high molecular emulsifiers, rheological properties are determined by the droplet size and emulsifier concentration [Schuchmann, 2005].

2.1.3 Destabilising Mechanisms

Emulsions are thermodynamically unstable systems, these systems tend to minimize the free energy [Kontogiorgos et. al., 2004]. In this case, the inner surface (interface) is reducing to the greatest possible extent, so that coalesce of oil droplets and phase separation takes place. The system is thermodynamically stable after complete separation of lipophilic and hydrophilic phase. This process is also referred to as emulsion breaking. There are several ways for the destabilization of an emulsion. The possibilities are subdivided into physical and chemical forces. The physical instabilities of emulsions include Ostwald ripening, creaming, flocculation, coalescence and phase separation [Rousseau, 2000]. For this work, chemical instability factors such as hydrolysis and oxidation of fat are less of interest [McClements, 2005].

Destabilisation mechanisms affect an emulsion never separately, but always in combination with other. The physical instabilities are illustrated in Figure 2.
Droplet aggregation (flocculation and coalescence)

Oil droplets in emulsions are always on the move, as long as the continuous phase or oil droplets have not been immobilized or adhered. Reasons for this movement are Brownian motion, gravity and/or mechanical forces [Walstra and de Roos, 1993]. The forces cause collision of the oil drops. Due to these collisions, the droplets remain separate from each other or aggregate. Mainly it depends on charge state and steric hindrance of the interfacial film of droplet particles, whether they attract or repel. The droplet aggregation is subdivided into two types of aggregation, Flocculation and Coalescence.

**Flocculation** describes the aggregation of two or more droplets, without the stability of droplets is influenced. Flocculation occurs if, attraction forces of droplets surfaces overlap the repulsive forces. Energetic interactions are also possible due to substances that have been absorbed by the oil droplet surface. There are two types of flocculates, in terms of their structures, open packing and closed packing. In the open structure, the droplets attach each other and remain in the position when they come in contact with each other without any subsequent displacement, forming flocculates with larger amount of continuous phase entrapped [Bremer et. al., 1993; McClements, 2005]. In close packing structure, droplets rearrange their position to each other after the first contact, therefore this structure is compact and only a very small amount of continuous phase entrapped [McClements, 2005]. Flocculation itself does not result into effective increase of the size of the oil droplets.
Nevertheless autoadhesion of droplets changed the droplet size distribution. The flocculation is a reversible mechanism of destabilization and the input of mechanical energy does the state reversed. Emulsions can be stabilized against flocculation, when oil droplet interface are electrostatically charged or by steric hindrance of the interfacial layer [Dickinson and Stainsby, 1988].

**Coalescence** is caused by a crack in the oil droplet interfaces. The crack is caused by collision of two drops, or develops from the flocculation. In contrast to the flocculation droplets remain individual are not intact but rather merge into one another [Danner, 2005].

**Creaming**

Creaming is a separation of the emulsion which is reflected in a layer of cream on the emulsion surface. The destabilisation process occurs due to the force of gravity and the density differences between the continuous and dispersed phase. In the case of O / W emulsion, the disperse phase has the lower density, which means that the gravitational force is directed upward, and therefore the oil droplets migrate upward in the emulsion [Timmermann, 2005]. Creaming is also a reversible mechanism of destabilization and the input of mechanical energy does the state reversed, because the oil droplet size remains unchanged. The effect of creaming is accelerated by the following factors, reducing the viscosity of the continuous phase and / or enlargement of the droplet size (coalescence) and / or aggregation of droplets (flocculation) [McClements, 2005].

**Ostwald Ripening**

Ostwald ripening based on the mass transport of the oil molecules of small droplets into larger oil droplet through the surrounding continuous phase. Oil diffuses from small droplets to larger droplets until it reaches a critical value (Kelvin instability), then the small oil droplet become unstable and disintegrate. Due to the mass transport, the volume of the large oil droplets increases and the total number of droplets is reduced. Furthermore is Ostwald ripening accelerated by the higher the solubility of the disperse phase. This destabilisation mechanism occurs only in O/W emulsions with small oil droplet diameter (nanoemulsion) [McClements, 2005].
2.1.4 Emulsifiers

Emulsifiers are the most important components of an emulsion. The amount and type of an emulsifier are decisive for the properties of an emulsion, because they determine the type and functionality of an emulsion. The rule according to Bancroft [1913] says, that phase in which the emulsifier can be solved better becomes the external/continuous phase. That means O/W emulsions have an emulsifier which has a better solubility in water. Furthermore, emulsifiers prevent a separation of the emulsion into its two original phases just after dispersing. Emulsifiers are also known as surface-active substances, because they reduce the interfacial tension of the phases and acts as a link between the water and the oil. This property is based on the amphiphilic character of an emulsifier. Emulsifier molecules have at least one group having a high affinity for non-polar phase and at least a group having affinity to the polar phase.

![Schematic representation of a phospholipid (lecithin) to the bounding surface area oil / aqueous phase.](image)

In an O/W emulsion, emulsifiers (Figure 3) are located at the interface, the hydrophilic (polar) group extends into the hydrophilic liquid (continuous phase) and the hydrophobic (non-polar) group extends into the oil (disperse phase) and together they form an interfacial film.
The interfacial film forms a mechanical and electrostatic barrier between the oil droplets and prevents the approximation between the droplets [Schubert, 2005].

The classification of emulsifiers can be based on different criteria, for example regarding to [McClements, 2005; Schuchmann, 2005]:

- charge of the hydrophilic groups
- lipophilic groups
- solubility in various solvents
- ratio of hydrophilic to lipophilic groups (HLB)
- rearrangement of the emulsifier molecules during the interaction with water
- crystal form

**Charge of the hydrophilic groups of the emulsifier**

The charge of the hydrophilic groups in the water determines whether an emulsifier has an ionic or non-ionic character. The ionic emulsifiers include three groups, these are divided into cationic, anionic and amphoteric emulsifiers. All three groups are surfactants and result in a reduction of surface tension and electrostatic stabilisation (by repulsion) of the emulsion [Matissek, 1979].

The amphoteric emulsifiers have anodic or cathodic characteristics depending on the pH of the aqueous solution. At the isoelectric point, the molecule has the properties of a zwitterion. Phospholipids are the most commonly used natural ampholytic emulsifiers [Moore, 1960].

**HLB (Hydrophilic-lipophilic balance) value**

Emulsifiers can be classified by the HLB value. The HLB value indicates the molecular weight ratio between the hydrophilic and lipophilic groups of an emulsifier. The HLB is an indicator for the affinity of an emulsifier to polar solvents (water phase) or non-polar solvents (e.g. oil phase). Emulsifiers having an HLB value close to 7 are soluble in oil and in water. An HLB value below 7 means, the emulsifier has a better solubility in oil and a value above 7 (e.g. lysophospholipids have an HLB 8-11) is a better solubility in water. The HLB value by using the Bancroft’s rule [1913] allows a better classification of emulsifiers with respect to the emulsion systems (O/W; W/O) [Griffin, 1949; Laughlin, 1981].
2.1.5 Emulsifying Properties of Proteins

Proteins are high molecular weight surfactants, due to their hydrophobic and hydrophilic areas. Their composition allows them to absorb with several parts of the molecule at the interface and to reduce the interfacial tension. The emulsifying property of a protein is limited by the factors solubility, hydrophobicity, and flexibility of the protein molecule in its spatial structure [Ternes et al., 1994]. The limiting factors are influenced by the physiological, characteristics molecular size, amino acid sequence, conformation and charge [Anton and Gandemer, 1997].

The **hydrophobicity** is dependent on the percentage ratio of hydrophobic groups to hydrophilic groups, and also influenced by the secondary and tertiary structure of the protein [Nakai, 1986]. It is also important to note that hydrophobicity affects not alone the efficiency of the emulsifier. The majority of the hydrophobic groups are in the interior of the protein. The hydrophobicity value is larger the more hydrophobic groups are involved in the adsorption. At the same time, the interfacial tension becomes on smallest. The literature therefore distinguishes between total and effective hydrophobicity [Ternes et al., 1994].

**Flexibility** means the ability of the protein molecules to spread on the droplet surface. The larger the covering interface, the greater the flexibility of proteins [Ternes et al., 1994]. The hydrophobic side chains of the protein molecule will lay open during the adsorption and thereby a larger contact surface area is formed onto the oil phase.

![Figure 4. Schematic representation of the adsorption flexible proteins at the oil / water bounding surface [Myers, 1991].](image)

Myers [1991] and Friberg [1997] developed the "trains-loop-tails" – model (shown in Figure 4) for surface-active polymers. This model describes the seizure of interface through flexible proteins.
The hydrophilic molecular components align themselves to the aqueous phase, while they form in the centre of the protein molecule "loops" and at both ends "tails". The hydrophobic segments are geared on the interface in the form of "trains". This process is known as interface denaturation's, because the tertiary structure of the protein is altered during unfolding [Dickinson and Stainsby, 1988]. The extent of the interface denaturation is determined by the ambient condition ion-concentration, temperature and pH-value [Walstra and de Roos, 1993]. The constitution of the interfacial film is dependent on protein concentration. A low protein concentration develops a flat interfacial film with elongated proteins. A high protein concentration forms a thick and dense interfacial film with compressed proteins, due to the compression of protein molecules towards the aqueous phase. Proteins occupy only a portion of the interface in comparison to the low molecular weight emulsifiers. Higher absorption efficiency of the protein molecules results from that less protein molecules are required to occupy the same interface, as with low molecular weight emulsifier molecules [Stang, 1998]. The boundaries surfaces which stabilized with proteins have a interfacial film with a higher viscoelasticity and thus also higher stability to mechanical stress [Nylander. and Ericsson, 1997].

**Solubility** of the proteins is influenced by the hydrophobicity and charge. The smaller the hydrophobicity is and the higher the charge of the protein side chains, the higher the solubility [Ternes et. al., 1994]. Furthermore, the charge of protein depends on environmental condition in which it is located. Ionic strength and pH determine whether the protein is anionic or cationic. In the range of the isoelectric point, the protein is present as a zwitterion molecule, and thus has low surfactant properties [Nylander, 2004].

2.1.6 Stabilisers

Stabilisers stabilise emulsion by increasing the viscosity of the continuous phase. The most important group of stabilizers are polysaccharides. The majority of the polysaccharides must be labelled as a food additive (such as xanthan or glycerol), whereas sugar (glucose, fructose or sucrose) are not considered as an additive [Kunz, 1993; Kunz and Frese, 2003]. As for example of a hydrocolloid, sugar binds water by hydrogen bonding of the continuous phase and increases the viscosity. The viscosity increase immobilised / decreased the moving speed of the fat droplets and the collision rate of the droplets are also reduced [Kunz, 1993]. Proteins as surfactants (biosurfactants) substances also have an influence on the physical destabilization mechanisms (2.1.3) as well as sugars. Water is immobilized by the adsorption of hydrophilic groups of protein and this leads to an increase in viscosity. The
formation of immobilizing network structures by hydrocolloids and proteins is dependent on temperature, pH, ion concentration and concentration of stabilizer [Knightly, 1968]. Furthermore, proteins form layer surrounding the dispersed droplets which stabilizes the emulsions by reducing the rate of coalescence [Walstra and de Roos, 1993].

2.2 Egg Yolk

2.2.1 Composition of the Egg Yolk

An egg contains about 36% yolk. The yolk itself is oil in water emulsion. This emulsion consists of 16% protein, 32-36% fat and 49-53% water [Ternes et. al., 1994; Ternes, 1994]. Minerals have a share of 1%. The content of carbohydrates is 1% and a quarter of them are bound to proteins. The lipids are composed of 66% of triglycerides, 28% phospholipids and 5% cholesterol. The majority of the fat is bonded to proteins and therefore is presented in lipoproteins [Ternes et. al., 1994]. The lipoproteins are subdivided into high-density lipoproteins (HDL) and low-density lipoproteins (LDL). There are still some further lipoproteins in yolk such as phosvitine, livetine and very-low-density lipoproteins (VLDL) [Guilmineua et.al., 2005]. Lipoproteins and phospholipids dominate the technological and functional properties of egg yolk.

Figure 5. Composition of the egg yolk (in dry matter) [Acker und Ternes et al., 1994].
Lipoproteins can be subdivided into different fractions according to their solubility. The yolk can be diluted with a sodium chloride solution and subsequently centrifuged. The two fractions consist of the insoluble sediment of the granules and the soluble supernatant plasma [McBee and Coterill, 1979]. Figure 5 shows the assignment of the above-mentioned lipoproteins fractions.

The **granules** are particles with a diameter from 1.0 to 1.3 µm which have an irregular polyhedral shape [Chang et. al., 1977]. The granule is composed of 64% protein, 30% lipids and 6% minerals. Their lipids consist of 54% phospholipids and 3.8% cholesterol [Ternes et. al., 1994]. The granules consists mainly of 60% HDL and 16% phosvitin and these are connected by calcium bridges to a stable structure. The structure is insoluble in a physiological solution (Ionic strength 0.16 M NaCl and pH-value 6.5-7). The granule starts to disintegrate at an ionic strength of 0.34 M sodium chloride. In this process, divalent calcium ions are replaced by the monovalent sodium ions. Then calcium bridges are broken up and phosvitin is partially removed. The complex of granule is dissolved completely at an ionic strength greater than 1.7 M sodium chloride. Furthermore, the granule complex includes 12% VLDL [Ternes et. al., 1994; Anton et. al., 2000a; Anton et. al., 2000b].

**Plasma** is the second fraction and it mainly consists of LDL micelles. LDL micelles are much smaller than the granules, and they have a spherical structure with a diameter of 20-60 nanometer. Water soluble livetine is the second substance in plasma [Chang et. al., 1977; Anton and Gandemer, 1997].

### 2.2.2 Proteins of Egg Yolk

The majority of yolk proteins are bound to lipids. Lipoproteins comprise polar lipids, triglycerides and proteins. This complex is stabilised by hydrophobic interactions between the hydrophobic regions of the alkyl (lipids), and the non-polar peptide chains. In addition, the lipoproteins are stabilised by ionic bonds between charged groups of the phosphatides and charged amino acid chains [Ternes, 1994; Belitz et. al., 2005].

**Granule Proteins**

**High-density lipoprotein (HDL)** is so named because of its higher density compared to other lipoproteins [Belitz et. al., 2005]. HDL has a higher density due to its high protein content (76%) and low lipid content (22%). Phospholipids have a 62% portion to the lipids
[Belitz et. al., 2005; Anton, 2007]. The structure of HDL consists of several globular folded protein chains which are embedded in lipids [partly in niches, partly at the surface] [Anton, 2007]. HDL has a high thermal stability in comparison to the other lipoproteins [Belitz et. al., 2005]. The higher thermal stability is probably based on the lipids and their type of connection (structure), if the lipids are separated the heat sensitivity is increased [Franzen et. al., 1970].

**Very-low-density protein (VLDL)** has a share of lipids up to 89%, this result in a very low density. The VLDL is very similar in its properties to LDL (plasma) and also has an almost identical to Apo protein [Ternes et. al., 1994].

**Phosvitin** is a water soluble glyco-phosphoprotein with a high content of phosphates. The protein consists of 10% of phosphorus, which represents 80% of the total phosphorus in the egg yolk. The phosphates are serine bound and half of the amino acids are phosphorylated. Therefore phosvitin has a higher hydrophilicity than any other lipoproteins [Belitz et. al., 2005]. Phosvitin has the highest thermostability of all lipoproteins being completely stable at 110°C for 10 minutes [Ternes et. al., 1994]. If the yolk once denatured, phosvitin is enveloped by the other clot [Ternes et. al., 1994].

**Plasma Proteins**

**Low-density lipoproteins (LDL)** consist of 84-90% lipids and about 66% of the lipids of yolk are localized in the LDL [Ternes, 1994]. The high lipid content causes the low density. LDL consists of 62% triglycerides, 22% phospholipids and 3% cholesterol and has a structure of a spherical particle with a diameter of 20-60 nanometer [Anton et. al., 2003]. The structure is described as LDL-micelle.

![Micelle structure of LDL](modified through Buxmann [2009]; [Kamat et. al., 1972; Anton et. al., 2003; Ternes, 2008]).
The micelle core built up as shown in Figure 6, of triacylglycerides and cholesterol esters which are surrounded by a membrane envelope of lipoproteins [Jolivet et al., 2006]. The membrane envelope is formed by a monolayer of phospholipids and Apo lipoproteins are embedded in this mono membrane [Anton, 2007].

**Livetine** are water-soluble globular protein fractions which can be divided into three subunits (α-, β- and γ-Livetine) [Belitz et. al., 2005]. Livetines have different thermal stability. The γ-livetin (62 °C) denaturises at first, then α-livetin (72 °C) and the β-livetin remains stable in structure up to 76 °C [Le Denmat et. al., 1999].

### 2.2.3 Lipids

#### Acylglycerols

Mono-and diglycerides are localized in the HDL with a proportion of 1.5 - 2.2%. Triglycerides are the main component of the lipids with a proportion of 66% and are predominantly present in the LDL [Ternes et. al., 1994].

#### Phospholipids

The lipids of the egg yolk consist of 28% of phospholipids. The phospholipids form the bulk of polar lipids in the egg yolk and they form the main lipid component of lipoproteins. Phospholipids are amphiphilic molecules, because they have at the glycerid skeletal structure (position -sn3) a polar head group (phosphoric acid or thereof esters) and two non-polar long-chain fatty acids (position -sn1 and -sn2). The phosphoric acid is esterified by an amino alcohol (serine, choline or ethanolamine) or other alcohols such as inositol or glycerol [Wieder, 1997]. The fatty acids of the phospholipids are mainly palmitic- (16:0), stearic - (18:0) and oleic acid (18:1). The unsaturated oleic acid is mainly located at the position -sn2 [Helmerich, 2004].

These phospholipids are soluble in fats, oils and in some polar solvents. Phospholipids form micelles in aqueous medium. The micelle formation depends on pH-value and temperature [Helmerich, 2004].
**Fatty Acids**

Fatty acids are amphiphilic molecules which have regions with hydrophobic and hydrophilic properties. Free fatty acids are W / O emulsifiers with an HLB value of 1 (oleic acid). Fatty acid salts (free fatty acids in connection with metal ions (Na⁺, Mg²⁺, Ca²⁺)) have properties of an OW-emulsifier having an HLB value of at least 18. The hydrolysis of lipids to free fatty acids causes a bitter taste in aqueous solutions / emulsions [Belitz et al., 2005; Jaekel and Ternes 2009].

### 2.2.4 Emulsifying Properties of Egg Yolk

The physical composition of egg yolk provides very good emulsifying properties due to the protein-phospholipid complexes (HDL und LDL). Furthermore, the emulsification is promoted by the structure (granule particles and LDL-micelles) of egg yolk and their technological properties. These capabilities permit the food industry to use of egg yolk as a natural emulsifier in O / W emulsions (salad dressings, sauces, mayonnaise) [Ford et al., 2004]. Recent works [Anton und Gandemer, 1997; Aluko and Mine, 1998; Le Denmat et al., 2000; Kiosseoglou, 2004; Nilsson et al., 2007] compared the individual fractions (plasma and granules) and their components. LDL (plasma); HDL (granules), Phosvitin (granules) and Livetine (plasma) were investigated with respect to their emulsifying properties. All examined constituents of yolk can adsorb at the emulsion interface and establish a stable film around the oil droplets. These components compete for a place at the bounding surface when they are present in abundance [Aluko and Mine, 1998; Nilsson et al., 2007].

**Emulsifying Properties of Egg Yolk Fractions**

**Plasma** has similar emulsification properties as the egg yolk, in contrast to granules [Le Denmat et al., 2000]. In other words, the emulsifying property of egg yolk is mainly characterised by the plasma fraction. LDL dominates the behaviour of the plasma [Anton et al., 2003]. Kiosseoglou and Sherman [1983] put forward the thesis for the adsorption of LDL during the emulsification process. The hypothesis says, LDL-micelle will separate during the adsorption of the O/W interface. The LDL-micelle splits into phospholipids, neutral lipids and Apo-LDL. Apo-LDL and phospholipids occupy the interface and the neutral lipids diffuse into the oil phase. Investigations of Mine et al. [1998] and Martinet et al. [2003] confirm this thesis of Kiosseoglou and Sherman [1983]. Phospholipids adsorb independently of the interface or
Apo-LDL has a higher absorption capacity at the interface than other soluble proteins [Le Denmat et al., 2000]. These better binding properties result from the structural design of the Apo-LDL. On the one hand there is the flexible structure, which allows a good adaptation to the oil droplets and on the other hand, the high hydrophobicity due to the spatial orientation of the main part of the hydrophobic groups. The properties confer a predominance of Apo-LDL in the adsorbing process and displace other proteins at the interface [Anton et al., 2003]. The emulsifying activity of livetins is described as low in the literature [Shenton, 1979] and they can be easily displaced from the interface by other emulsifiers / proteins.

**Granule** has a very low solubility in its natural environment (Egg yolk: pH-value of 6.5 to 7 and / or ionic strength less than 0.3 M sodium chloride) due to that they tends to aggregate. The solubility of the granules is significantly increased if the pH-value is set higher than 7 (and / or the ionic strength is set to be higher than 0.3 M NaCl) and thus the calcium bridges are broken. This results in the release of phosvitin and HDL [Causeret et al., 1992]. The emulsifying capacity is improved by adsorption of Apo-HDL to the interface [Anton and Gandemer, 1997; Aluko and Mine, 1998; Le Denmat et al., 2000]. The phosvitin also has good emulsifying properties regarding coalescence and creaming [Chung and Ferrier, 1992].

Apo-HDL and Apo-LDL are quantitatively distributed equally at the interface. Apo-HDL occupies only a small part of the interface due to its compact and inflexible structure. For this reason, it has only a small influence on the emulsifying properties [Le Denmat et al., 2000].

### 2.2.5 Thermal Behaviour of Egg Yolk Proteins

The thermal denaturation of egg yolk proteins begins at a temperature between 65 °C and 70 °C. The egg yolk proteins (granule and LDL-micelle) denature to their second structure and form with one another a three-dimensional protein network which increases the viscosity. The LDL-micelle has the largest proportion of gelled network [Kojima and Nakamura, 1985; Le Denmat et al., 1999; Kiosseoglou, 2004].
Proteins of the LDL-micelle denature at a temperature above 65 °C. These proteins when unfolding interact with each other and form hydrogen bonds, ionic bonds and hydrophobic interactions and aggregates to a gel [Le Denmat et al., 1999]. Livetins are also sensitive to high temperatures. γ- and α-livetin are stable up to a temperature of 62 °C or 72 °C and the β-livetin shows no change up to 76 °C [Le Denmat et al., 1999]. The investigations of Le Denmat et al. [1999] and Guilmineau et al. [2005] proved a relatively high thermal stability (up to 76 °C) of HDL. The native granule particles show the same properties of protein denaturation than HDL, but the particles do not aggregate with each other to form a gel network [Anton et al., 2000]. Phosvitin is the most stable heat protein, it does not show any change when processed at a temperature of 110 °C for 10 minutes [Ito et al., 1983]. Recently, the food industry is using mainly phospholipase A2 (PLA2) to increase the heat stability of egg yolk proteins.

2.3 Thermostable (enzyme modified) Egg Yolk

2.3.1 Enzymatic Modification by Phospholipase

Enzymes are an effective way for changing the technological properties of foodstuffs. Phospholipase type A (PLA) is an enzyme which is used since a long time to alter egg yolk and its properties [Dutilh and Groger, 1981; Reimerdes, 2005].

![Figure 7. Cleavage sites of phospholipases on the phospholipid molecule [Markert 2004].](image)

Phospholipases belong to the class of hydrolases, they cleave by means of hydrolysis ester compounds of the phospholipids [Markert, 2004]. There are four possible sites of action (hydrolysis sites) of phospholipases in a phospholipid. Phospholipases are classified according to their place of activity (Figure 7). Phospholipase A1 (EC 3.1.1.32), and phospholipase A2 (EC 3.1.1.4) belong to the alkyl hydrolases, because they cleave the ester
compounds sn\textsubscript{1-} or -sn\textsubscript{2-} of fatty acids. Phospholipase B is also an alkyl hydrolase because it hydrolyse both ester compounds sn\textsubscript{1-} or -sn\textsubscript{2-}. Phospholipases C (EC 3.1.4.10) and D (EC 3.1.4.4) are allocating in the group of phosphodiesterases. Markert [2004] describes in their work, the phospholipase C hydrolyse phospholipids to 1.2 - diacylcholin and phospholipase D cleaves the phosphodiester compounds from the alcohol group.

2.3.2 Hydrolysis Mechanism of Phospholipase A\textsubscript{2} (PLA\textsubscript{2})

Phospholipase A\textsubscript{2} mixed with industrial egg yolk (phospholipids) results in lysophospholipids and free fatty acids. The PLA\textsubscript{2} catalyzes the hydrolysis of phospholipids to lysophospholipids and free fatty acids which is a complex procedure at the molecular level. The sequence of the hydrolysis can be broken down and simplified into three stages. In the first the enzyme-substrate complex stage is formed. The fatty acid residue of phospholipid molecule binds to the hydrophobic channel of active centre of PLA\textsubscript{2}. The carbonyl group (from the phospholipid) of the ester compound (sn\textsubscript{2-}) to be broken down is stabilized at the amino acid residue of the enzyme. The stabilisation is affected by calcium ions. It causes electrostatic interactions such as hydrogen bridge bonds and ionic interactions between the carbonyl group and the amino acid residue (PLA\textsubscript{2}). The second step leads to the cleavage of the ester compound by a nucleophilic attack of the hydroxyl residue. In the final step, water molecules reach the active centre and displace the fission products (lysophospholipids and free fatty acids). [Markert, 2004]

2.3.3 Changes in the Properties of Egg Yolk by the Hydrolysis with Phospholipase A\textsubscript{2}

The hydrolysis with phospholipase A\textsubscript{2} changes the phospholipids of egg yolk, and thus the technological properties of whole egg yolk.

- The emulsifying activity of egg yolk is increased [Daimer and Kulozik, 2009]
- The heat stability of yolk stabilized emulsions is increased [Van Dam, 1974]
- The viscosity of emulsions is increased [Dutilh and Groger, 1981]

The hydrolysis of phospholipids is only a small change in egg yolk matrix. However, it changes the whole structure of egg yolk. The granule particles are partially broken up, thereby granules has a higher solubility and an increased emulsifying properties [Daimer and
Kulozik, 2008]. The PLA$_2$ modified granules shows no proof of a better heat stability of O/W emulsions [Buxmann et.al., 2008].

PLA$_2$ hydrolysis shows no change in solubility and emulsifying properties of the plasma [Daimer and Kulozik, 2008]. Emulsions prepared with PLA$_2$-modified plasma showed significantly improved heat stability. This property results from the complex formation of Apo-LDL, lysophospholipids and free fatty acids [Mine, 1997]. Furthermore, PLA$_2$ enzymes prevent a partially thermal gelation of egg yolk [Daimer and Kulozik, 2008]. The proteins denaturation of proteins occurred during heating is slightly improved by the enzymatic modification, protein structures will be protected against a completely unfolding and loss of technological properties. This effect is based on the previously described complex formation (Apo-LDL, lysosphospholipids and free fatty acids).

2.4 Homogenisation Processes in Industry

2.4.1 Homogenisation Systems

**Rotor-stator systems**

Rotor-stator machines are mostly simple construct’s and therefore easy to maintain and inexpensive in acquirement. The crushing effect and the energy input are provided by the rotating parts. There are rotor-stator systems for different production volumes (laboratory scale up to production scale).

Figure 8. Example of a discontinuous working rotor-stator system [McClements, 2005].
They are operated in a discontinuous (shown in Figure 8) or semi-continuous process. These systems can be used flexibly in contrast to other homogenization systems, because they enable different process steps in one single apparatus, such as temperature management (pasteurisation and / or cooling), mix of ingredients and emulsifying. Emulsification processes are usually characterized by a large average droplet diameter ($x > 2 \mu m$) and a wide droplet size distribution. This takes place due to unfavourable distribution of a low energy input in a wide and non-uniform crushing zone [Anbarci, 1987].

![Figure 9. Composition of a colloid mill [McClements, 2005].](image)

Colloid mills (Figure 9) and gear-rim disperser are continuous operated rotor-stator systems. They could transfer in a short time a great energy into the medium / product. The colloid mill generates turbulent flows in its dispersing zone between the stator and rotor. Turbulent flows also generates by gear-rim dispersers for crushing of droplets (Figure 13). The flow is generated by coaxial interlocking rings which are equipped with different large radial openings [Armbruster, 1990].
Ultrasonic Systems

Figure 10. Schematic of a discontinuous working ultrasonic system [Schuchmann and Danner, 2004].

Ultrasonic systems operate as discontinuous or continuous processes (Figure 10). They produce finely dispersed, highly liquid emulsions. Crushing mechanism is generated by cavitation (sonotrode) which results in micro turbulence (Figure 13). Based on a very high level of product stress a small mean droplet diameter (down to 0.4 μm) is obtained [Behrend and Schubert, 2001].

Membrane Emulsifying

Figure 11. Schematic principle of membrane emulsification [Leal-Calderon et. al., 2007].

Membrane emulsification is a continuous process in which the dispersed phase is forced through the pores (illustrated in Figure 11) of a porous micro ceramic or glass membrane. Continuous phase flows along the pore outlet and encloses small shaped droplets. This emulsification process enables producing of small droplet diameter (down to 0.2 μm) with a
narrow droplet size distribution. Furthermore, the stress of product is very low because the droplets are produced directly and not with energy of currents (99% of fluid flow energies are converted into heat). Emulsions with a high proportion of disperse phase must be recirculated, and that increases considerably the cost of production [Schuchmann, 2005].

**High-Pressure Systems**

All high-pressure homogenizers consist of two basic elements: high-pressure pump (piston / intensifier) and dispersion unit (homogeniser valve / nozzle). The high pressure pump produces the pressure / energy, which is expanded in the homogenising nozzle and leads to a smaller droplet size. High pressure systems achieve a pressure of 100 up to several 1000 bar and they operate in a continuous process with a high product flow. Depending on the pressure and homogenising nozzle a small mean droplet diameter (down to 0.2 μm) can be achieved. The homogenising nozzle affects the shape of the droplet-reducing flow. Significant influence has the design and gap width of the nozzle.

![Different types of high-pressure homogenisation valves](image-url)

Figure 12. Different types of high-pressure homogenisation valves [Stang et. al., 2001].
Dispersion unit can be subdivided into radial-diffusers, counter-jet-dispergator and axial flow-nozzle-systems (illustrated in Figure 12). Forces of droplet disruption depend on the type of dispersion unit. Generally it can be said that turbulent flows are prevalent with forces of shearing and inertia in the dispersing zone. Counter-jet-dispergators and axial flow-nozzle-systems generates laminar extensions flows and radial-diffusers generates cavitation’s and impacts (Figure 13). Additional droplet size disruption forces are high-speed impacts and shock waves. However, the product stress is enormous due to the flow rates and the high pressure gradients [Stang et. al., 2001].

![Figure 13. Summary overview of various methods for emulsification [Schultz et. al., 2004].](image)

### 2.4.2 Ultra-High Pressure Homogeniser “FPG11300 Hygenic Homogeniser”

#### General

Novel generation of high-pressure homogenisers (HPH) reaches pressure level 10 - 15 times higher pressure than traditional machines. Commercial manufacturers of ultra-high pressure homogeniser (eg: Avestin®, Invensys™ AVP, Microfluidics™, Stansted Fluid Power Ltd and Niro Soavi S.p.A.) generate a maximum pressure 350 to 500 MPa. The manufacturers also specify that their homogenisers reduced the number of microorganisms in product by cell destruction [Popper and Knorr, 1990; Diels et. al., 2004]. In addition, ultra-high pressure
changed the product matrix of emulsions much more effective than traditional homogenisers. Droplet (particle or micelle) size is substantially reduced and it is possible a narrower droplet size distribution. Furthermore, nanoparticles and nano-suspensions, can also be manufactured.

**Construction Stansted Fluid Power**

![Diagram of FPG11300 Hygenic Homogeniser](image)

Figure 14. Simple scheme of the structure of “FPG11300 Hygenic Homogeniser” [Handbook FPG11300, 2011].

The homogenizer system “FPG11300 Hygenic Homogeniser” of Stansted Fluid Power LTD is composed of an external supply unit and a homogeniser (illustrated in Figure 14). The supply unit is for reasons of hygiene outside of the homogeniser, because hydraulic pressure is created with oil, which is not suitable for food products. The pump generates up to 20 MPa and transmits this pressure to two intensifiers /pistons in homogeniser. Actual homogenizer consists of a positive displacement pump (1.5 l / min), which supplies two intensifiers with
products /fluids. The pressure is set at the first homogeniser valve (up to 350 MPa), the narrower the gap between homogenising valve and valve seat, the higher the pressures will be generated. The pressure decreases after first valve to the adjusted pressure level of the second homogenising valve (up to 10 MPa). After passing the last homogenising valve the ambient pressure occurs. The homogeniser system provides the opportunity to cool down the product with an external cooling unit. Cooling of product is carried out on track between two homogenising valves [Handbook FPG11300, 2011].

Temperature of the product during homogenisation

Knorr [1999] determined the temperatures arise in the product during the homogenisation process. The intensifier causes the pressure to rise quickly in the process fluid, these results in a temperature increase of 3 °C/100 MPa. The major temperature increase in the process fluid is caused by the forces during the pressure drop acting in the homogenising valve gap

Figure 15. Schematic representation of the effect of ultra-high pressure on the product temperature [Donsi et al., 2009].
Temperature increases depend on composition and viscosity of process fluids. Schematic diagram in Figure 15 shows the correlation between pressure and temperature.

Homogenisation Valves

Figure 16. Schematic representation of the ultra-high pressure needle valve system (modified after Floury et al [2004a and 2004b])

Selecting the proper homogenizing valve is decisive for the pressure level of a homogenizer. Stansted Fluid Power LTD used in the first pressure stage a kind of radial-diffuser in combination with needle valve (Figure 16). This valve can withstand very high pressures due to its aerodynamic shape. The robust ceramic needle valve is resistant to the self-generated highly turbulent and cavitating flow and also against the resulting energy. The latter may generate serious damage to the valve gaps, by fast-growing and/or condensing gas bubbles [Treiber, 1979]. In the second pressure stage an ordinary flat valve (Illustrated in Figure 12 and Figure 15) is installed. This has to withstand only low compression forces and self-generated turbulence. Agglomerates which might result from the first pressure stage are disrupted by flat valve [Pereda et. al., 2006].
2.5 State of Science - influence of high pressure homogenization on the properties of emulsions and their components

In recent years there have been some studies on the effect of high pressure homogenization (HPH) on proteins. Heinzelmann et al. [1994] noted, treatment of egg white and soy beans proteins resulting in an increased foam ability and an increased water binding capacity of fava bean protein. Floury et al. [2002] examined in particular, the impact of HPH to soy protein-stabilized emulsions. They discovered HPH partially denatured proteins, thereby decreased the droplet size in emulsions. Furthermore, proteins can form gel-like network structures due to hydrophobic interactions with each other. In a previous work, Floury et al. [2000] have examined the impact of HPH (20; 150; 300 MPa) on droplet size distributions and rheological properties of model oil-in-water emulsions. The investigation revealed that increasing pressure leads to a progressive increase of viscosity and shear thinning behaviour of emulsion. The shear thinning behaviour is caused by the formation of clusters or aggregates of droplets (flocs), flocs deforms by increasing shear stress until they will completely destroyed (Figure 17). In addition they describe, the pressure level has no significant influence on droplet size distribution.

![Figure 17. An emulsion containing flocculated droplets exhibits shear-thinning behaviour because the flocs are progressively aligned, deformed and disrupted in the shear field [McClements, 2005].](image)

The most recent work in this field is by Marco-Molés et al. [2012]. This investigation of the egg yolk / milk stabilized emulsion shows an aggregated lipoprotein network, while reducing the proportion of water-soluble proteins and an increased coalescence of oil droplets at a pressure level of 100 MPa. The pressure range of 150 MPa caused an increase in the oil droplet size because of denatured proteins (which lead to protein-protein interactions (aggregates)). Therefore, there was a lack of proteins to stabilize the newly formed interfaces. The ultra-high pressure homogenized emulsion with 250 MPa was not stable due
to phase separation. This phase separation soon after production has been caused by the combination of high-pressure homogenization and the resulting high temperatures or to put it more succinctly, due to the complete destruction of the conformation of proteins. Marco-Molés et al. [2012] also investigated changes of characteristics of the treated emulsions during storage (1 week at 4 °C). They came to the conclusion that the characteristics of each emulsion does not significantly change during storage.

3. Materials and Methods

3.1 Material

Material:

Fluorescent dye, Alexa Fluor 555 Fluka
Fluorescent dye, FITC Fluka
Liquid egg yolk (homogenised und pasteurised) Ovobest
Sunflower oil VEGOLA Netto
Thermo stable (PLA2) liquid egg yolk Ovobest

Chemicals:

Formic acid Merck
Butan-1-ol Merck
Carboxymethylcellulose (CMC) Merck
Imidazol Merck
Kieselgur, Celite 545 Sigma-Aldrich
Miglyol 812 N Sasol
Natriumdihydrogenphosphat Merck
Petroleum spirit Riedel
Potassium hydroxide pellets (iso-Octan)  Riedel
Sodium chloride p. A.  AppliChem
Sodium hydroxide  Fluka
Sodium lauryl sulfate (SDS)  Roth

Equipment:
Centrifuge RC-6+ (Rotor F18-12x50)  Sorvall
Centrifuge tube from polycarbonate (30 ml)  Thermo
Confocal Laser Scanning Microscope (ECLIPSE C1)  Nikon
Cooling device SCE 9.0 V  ERS
Digital camera D70  Nikon
Displacement pump BC9B  Seeppex
Droplet-Volume-Tensiometer DVT 10  Krüss
Extraction unit B-815  Büchi
Folded filter S&S 595  Marchery-Nagel
FPG11300 Hygenic Homogeniser  Stansted
Gas chromatograph B-820  Büchi
He/Ne- und Ar-Ion-Lasersystem (CLSM)  Melles Griot
Hydro 2000S  Malvern
Hydrophobic filter MN 616 WA  Marchery-Nagel
Laboratory Scale PE 3600  Delata Range
Laboratory-type drying cabinet  Memmert
Laser diffraction spectrometer (Mastersizer 2000)  Malvern
Magnetic Stirrer Plate, MR Hei Standard  Heidolph
pH meter 765 Calimatic  Kniek
3.2 Methods

3.2.1 Ultra-High Pressure Homogenization of Egg Yolk

Preparations

In the trials, two types of egg yolk are ultra-high pressure treated. The first type, liquid egg yolk (OVOBEST Eierprodukte GmbH & Co. KG), which is a traditional homogenized and pasteurized liquid egg yolk. And the second type is thermo stable (PLA₂) liquid egg yolk (OVOBEST Eierprodukte GmbH & Co. KG) which is the product of liquid egg yolk (Type 1) modified enzymatically by PLA₂. Both types of egg yolk are diluted (1:1) with physiological 0.17 M sodium chloride (AppliChem GmbH) solution in bidistilled water to an egg yolk solution. The yolk solution is made using a propeller stirrer for 45 minutes.

Ultra-high pressure homogenization

The diluted egg yolk tempered to around 2 °C is pumped by means of the displacement pump BC9B (Seepex, Bottrop) in the feed tank into the FPG11300 Hygenic Homogeniser from Stansted Fluid Power LTD (refer to 2.4.2). The intensifier compresses the yolk solution and the fluid is pushed through a pre-set homogenization gap. Product is cooled (counter current) on the line between the two homogenizing valves. Cooling feeds the external cooling device SCE 9.0 V (ERS, Sraßenhaus) with 5 °C cold water. After the second homogenizing valve, the (so-called) Premix is packed and stored in the fridge until the analysis or preparation of an emulsion. The complete production process of a Premix is illustrated in Figure 18.
3.2.2 Method for Producing O/W-Emulsions

The manufacture of emulsions is used for the characterization of the changes in the yolk properties (emulsifying and stabilizing ability) after high pressure homogenization processing. The O/W emulsions consist of the ingredients of ultra-high pressure treated or untreated Premix (unmodified or modified PLA₂), sunflower oil (VEGOLA, Netto-Marken Discount AG & Co.KG) and a physiological solution of sodium chloride (mentioned in 3.2.1). For each emulsion is produced a 200 ml Pre-Emulsion. 150 ml of the Pre-Emulsion is dispersed to an emulsion and the remaining 50 ml of Pre-Emulsion is discarded. The exact process of emulsion preparation is shown in the flow diagram (Figure 19). The standardization of all emulsions takes place via the ratio of oil phase to aqueous phase and the protein content in the aqueous phase.
Figure 19. Production steps for preparation of O/W Emulsions

**Pilot-Tests 1**

The O/W emulsions of the Pilot-Tests 1 are used to establish the stability to pressure level of the both variants of egg yolk (PLA\(_2\)-modified yolk and unmodified egg yolk). In Table 1 is shown the recipe for the preparation of O/W emulsions. All examinations of the samples performed the day after their preparation.

Table 1. Pilot-Tests 1: Composition of the O/W emulsions of unmodified egg yolk as well as modified PLA\(_2\) egg yolk (O/W; 50/50 emulsion with a protein content of 1.25% in the aqueous phase).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Premix</td>
<td>20 g</td>
</tr>
<tr>
<td>0.17 M NaCl-Solution</td>
<td>80 g</td>
</tr>
<tr>
<td>Sunflower Oil</td>
<td>100 g</td>
</tr>
</tbody>
</table>
Pilot-Tests 2

In the Pilot-Tests 2 different recipes / emulsions are prepared with Premix of PLA$_2$-modified egg yolk. Recipes (Table 2) vary in the percentage composition of its components. The aim of the Pilot-Tests 2 is to determine the recipe / emulsion, which shows the biggest influence of ultra-high pressure homogenisation to the enzyme (PLA$_2$) modified yolk. All examinations of the samples performed at day 1 and 10 after their preparation.

Table 2. Pilot-Tests 2: Composition of the O/W emulsions of PLA$_2$-modified yolk (O/W; 50/50, 35/65 or 10/90) emulsion with a protein content of 1.25% or 1.625 in the aqueous phase.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>1.25%</th>
<th>1.25%</th>
<th>1.25%</th>
<th>1.625%</th>
<th>1.625%</th>
<th>1.625%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premix</td>
<td>20 g</td>
<td>20 g</td>
<td>20 g</td>
<td>26 g</td>
<td>26 g</td>
<td>26 g</td>
</tr>
<tr>
<td>0.17 M NaCL-Solution</td>
<td>160 g</td>
<td>110 g</td>
<td>80 g</td>
<td>154 g</td>
<td>104 g</td>
<td>74 g</td>
</tr>
<tr>
<td>Sunflower Oil</td>
<td>20 g</td>
<td>70 g</td>
<td>100 g</td>
<td>20 g</td>
<td>70 g</td>
<td>100 g</td>
</tr>
</tbody>
</table>

Main-Tests

On the basis of the results of Pilot-Tests 2, the recipes for the emulsion are modified for the analysis of the Main-Tests. The emulsion is prepared according to the recipe of the Table 3. All examinations of the samples performed the day after their preparation.

Table 3. Main-Tests: Composition of the O/W emulsions of PLA$_2$-modified egg yolk (O/W; 50/50 emulsion with a protein content of 2% in the aqueous phase).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>32 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premix</td>
<td></td>
</tr>
<tr>
<td>0.17 M NaCL-Solution</td>
<td>68 g</td>
</tr>
<tr>
<td>Sunflower Oil</td>
<td>100 g</td>
</tr>
</tbody>
</table>
3.3 Analytical Methods for Characterization of Egg Yolk

3.3.1 Dynamic Interfacial Tension

Principle:

The number of droplet splits is counted in a given volume of liquid. The droplet-tensiometer measures dynamic interfacial / surface tension at the interface (liquid / liquid), while new interfaces are formed. Dynamic means, that determination of the surface tension (SFT) is effected in function of the droplet formation time (“Average SFT vs. Average Surface Age”). Therefore it can be said, the more effective an emulsifier, the lower the interfacial tension with respect to the surface age.

Execution:

The Droplet-Volume-Tensiometer DVT 10 (Krüss GmbH, Hamburg) investigated the interfacial behaviour of the Premix (PLA₂ modified and unmodified). Oil (Miglyol 812 N, Sasol) is pumped into the aqueous phase (diluted Premix 0.5% w / w) with defined volume flow at 20 °C.

The time is measured from the growth of the drop to the detachment of the capillary. The volume of the droplet is direct proportional to the interfacial tension between the two phases (Oil to Premix). The droplet volume is determined by the time interval between two successive drops of oil. Time is set in relation to the volume flow. The surface tension ($\sigma_i$) is determined according to the equation 1. Volume of each droplets is detected in reference to the time ($V = 0$) until droplet separation at a known flow rate of oil.

$$
\sigma_i = \frac{V_{Droplet} \cdot (\rho_H - \rho_L)g}{\pi \cdot d}
$$

(Equation 1)

$\rho_H$: density of the aqueous phase; $\rho_L$: density of the oil phase; $V_{Droplet}$: droplet volume; $d$: capillary; $g$: gravitational acceleration

3.3.2 Scanning Electron Microscopy (SEM)

The scanning electron microscope (JEOL, Japan) with a Cryo-Preparation unit is used to visualize the microstructure of the yolk.
Sample preparation:

Approximately 1.5 \( \mu \text{l} \) Premix are frozen in super cooled liquid nitrogen (-196 °C). The high temperature gradient causes the ice crystals are formed only a few nanometres in size, and thus much smaller than the visible structural elements. Subsequent to the cryogenic freezing, the sample is broken at -180 °C and the water is removed at the sample surface at -40 °C by sublimation. The sample is then coated with gold and examined at -180 °C in the SEM.

Execution:

In the measurement, the sample surface is scanned with a high energy electron beam in an isometric pattern. The emitted secondary electrons are detected and calculated by software.

3.4 Analytical Methods for Characterization of O/W Emulsions

3.4.1 Rheology

Principle:

The sample is analysed by sheared in a defined gap. This gap is adjustable by the mutually movable measuring body (cone, plate). The emulsion are a non-Newtonian fluid, and therefore are defined as the ratio of shear stress \( \tau \) and shear rate \( \gamma \) and is a measure of the internal friction resistance which opposes the deformation.

Execution:

The rheological characterization is performed with the rotational rheometer AR 2000 (TA Instruments, Alzenau) using a cone-plate measuring system. The cone (\( d = 50 \text{ mm} \)) is arranged coaxially, and the plate has an opening angle of \( \varphi = 2 ^\circ \). Receiving the rheograms performed at increasing shear rate in the range of \( \gamma = 0.1 \text{ s}^{-1} \) up \( \gamma = 1000 \text{ s}^{-1} \) and the temperature of the emulsion is 20 °C. Thereby, 81 individual values are taken (run-up) for the rheological curve.
Evaluation:

For characterization of flow behaviour of emulsions is the power law model used by Ostwald / DE WAELE (Equation 2)

\[ \tau = K \cdot \left( \frac{\dot{\gamma}}{\dot{\gamma}_0} \right)^n \cdot \dot{\gamma}_0 \cdot 1 \text{s}^n \]  

(Equation 2)

\( \tau \): shear stress, \( K \): flow consistency index, \( \dot{\gamma} \): shear rate, \( n \): flow behaviour index

In this model, the viscosity \( \eta \) of the sample will be described at a certain shear rate \( \gamma \) by the product-specific characteristics flow consistency index \( K \) and flow behaviour index \( n \). Using linear regression, the flow curves were analysed with the software "RheoStar 5.0" [Franke und Tscheuschner, 1995]. The flow consistency index \( K \) is a direct measure of firmness of the emulsion, while the flow behaviour index \( n \) describes the deviation from Newtonian (\( n = 1 \)) behaviour. In the range of \( 0 < n < 1 \) exists the relevant pseudoplastic (shear-thinning) behaviour of emulsions.

3.4.2 Stability Tests

Principle:

The stability test is a visual analysis of an emulsion during a defined period of time. It will determine the stability of the emulsion with respect to creaming and phase separation.

Execution:

The emulsion is directly after preparation (day 0) placed into a 20 ml test tube covered with aluminium foil. These tubes are stored in a refrigerator at 7 °C. Using photos (digital camera D70, Nikon) the change of the emulsion is documented. The study period extended for 14 days and images were taken on day 1 (24 h) after production (a.p.), day 3 a.p., day 7 a.p., day 10 a.p., day 14 a.p.. For the Pilot-Test 1 samples, the stability method was used following a demanding criteria of stored at room temperature (25 °C). This measurement reassures the stability at room temperature without refrigeration conditions as required by certain of customer and marketable conditions. The method only differed in storage in the temperature.
3.4.3 Droplet Size Distribution and Surface to Volume Ratio

Principle:

The laser diffraction spectrometry is a fast method for determining the oil droplet size distribution and the surface to volume ratio (also called specific surface area) of an emulsion. The sample is irradiated in the measurement cell with a laser beam. The oil droplets change the direction of the incident laser beam and it results in the refraction, reflection and diffraction of light. The resulting diffraction pattern of light is then mathematically converted with the optical model (Fraunhofer-theory). The diffraction angle and the scattering intensity of light is a measure of oil droplet size.

Execution:

For the characterization of emulsions, oil droplet size distribution and specific surface area are determined using the laser diffraction spectrometer Mastersizer 2000 (Malvern Instruments). The emulsion is diluted before the measurement in a polar medium (Sodium lauryl sulfate; Roth) at a ratio of about 1:20000. The diluted sample is placed in the measuring device Hydro 2000S (Malvern Instruments), until the predetermined obscuration has been reached.

Evaluation:

For the description of particle size distribution, the mean average droplet size $x_{50}$ specified in addition to percentiles of $x_{10}$ and $x_{90}$ are determined. They represents 10%, 50%, 90% of all particles in collective having a diameter less / equal to this value. These parameters are descriptive in a monomodal distribution function. The surface-related diameter $x_{3,2}$, also known as Sauter mean diameter (Equation 3) allows calculation of the total surface area of the dispersed oil droplets in all classes.

$$x_{3,2} = \frac{\sum N_i x_i^2}{\sum N_i x_i^2}$$  \hspace{1cm} (Equation 3)

$N_i$: number of oil droplets; $x$: diameter of oil droplets; $i$: size range of oil droplets
The Sauter mean diameter is to be used if the surface to volume ratio $S_v$ (Equation 4) of the dispersed phase is to be calculated. The interface of oil droplets is in the following ratio to volume:

$$S_v = \frac{\pi x^2}{\pi x^3/6} = \frac{6}{x_{3,2}}$$  \hspace{1cm} (Equation 4)

Thereby means a large surface to volume ratio an emulsion with small oil drops and vice versa. In addition, the dispersion index $SPAN$ (Equation 5) is implied in evaluation as a factor for the width of oil droplet distribution.

$$SPAN = \frac{x_{90} - x_{10}}{x_{50}} = \frac{d_{0.9} - d_{0.1}}{d_{0.5}}$$  \hspace{1cm} (Equation 5)

### 3.4.4 Protein-Interface-Occupancy

Proteins have besides low molecular weight emulsifiers surfactants an important function as recipe components in emulsions because they influence the stability of emulsion and thus also are involved in the construction and the microstructure of the emulsion.

#### 3.4.4.1 Separation of the Continuous and Disperse Phases

The phase separation is carried out by centrifugation (centrifuge RC-6+ with rotor F18-12x50, Sorvall) of 30 g of emulsion at 10,000 g for 30 min at 20 °C. The settled aqueous phase was skimmed and the cream layer is re-suspended in 20 mM imidazole buffer (pH 7) to 30 g. This re-suspended cream is centrifuged again under the same conditions. The cream phase and the aqueous phase are stored for the following analysis.

#### 3.4.4.2 Determination of Protein Content of Dispersed Phase

**Principle:**

Protein content is determined by the amount of nitrogen in the sample. The sample is combusted (up to 1200 °C) in a crucible, and released nitrogen is detected at the nitrogen detector.
Execution:

Washed disperse phase is pre-dried (laboratory-type drying cabinet, Memmert) for 1 hour at 110 °C, then 0.6 g of sample is weighed into a measuring crucible. The nitrogen detection is fully automatic by analyser (Vario Max PM 1038; Elementar Analysensysteme). The protein content (g/100 g) is calculated from the detected amount of nitrogen and the Kjeldahl-factor.

3.4.4.3 Calculation of Protein-Interfaces-Occupancy of Emulsions

The interface occupancy \( \Gamma (\text{mg} \cdot \text{m}^{-2}) \) is derived from surface to volume ratio \( S_v \) (3.4.3) of the emulsion \( (\text{m}^2 \cdot \text{g}^{-1}) \) and the protein concentration \( e_F \) (3.4.4.2) of its dispersed phase \( (\text{mg} \cdot \text{g}^{-1}) \). The calculation of the interfacial occupancy \( \Gamma \) is performed according to following Equation 6.

\[
\Gamma = \frac{e_F}{S_v}
\]  

(Equation 6)

3.4.5 Extractable Fat Fraction (EFF)

Principle:

Definition of extractable fat means the portion/fraction of fat, which is extracted with organic solvents of an emulsion. In this context can be said, the more stable and closer the interfacial film of emulsion, the less solvent can penetrate the dispersed phase. As a result, less fat is extracted from the oil drops and the total amount of extractable fats decreases.

Execution:

In the first step, 10 g of emulsion are mixed with 50 ml of petroleum spirit in a 250 ml Erlenmeyer flask. This flask is moved 5 min on the shaker (180 rpm) and then the petroleum spirit is decanted and filtered through the hydrophobic filter (MN 616 WA; Marchery-Nagel) in a folded filter (S&S; Marchery-Nagel) into a round bottom flask. In the second step, the solvent is removed at 60 °C in a rotary evaporator (011, Büchi; 750-200 mbar). Then the flask is dried at 103 °C in a laboratory drying cabinet and 45 min cooled in a desiccator. After the cooling process, the flask is weighed on an analytical balance. In the third step, the extractable fat fraction (EFF) is calculated using the following Equation 7.
$$EFF = \frac{m_0 \cdot 100 \cdot 100}{\omega_F \cdot m_1}$$  \hspace{1cm} \text{Equation 7}

$m_0$: emulsion weighed in g; $m_1$: mass extracted fat after drying in g; 
$\omega_F$: total fat content of the emulsion in% 

### 3.4.5.1 Determination of total Fat Content of Emulsion

The total fat content of emulsions was determined in accordance with the DGF Caviezel ® standard method C 19-III (00) A.

**Principle:**

The fat from the emulsion is extracted by the addition of an internal standard (C 19-III (00) A). After extraction, the potassium salts are converted into the free fatty acids and determined by gas chromatography the proportion of fatty acids. The fat content is specified as triglyceride content in 100g sample and calculated by means of response and conversion factors to the calibration standard curves.

**Execution:**

The emulsion (1g) is weighed with an internal standard (0.5 g) into the extraction unit B-815 (Büchi, Switzerland). Later, 1.5 g of potassium hydroxide pellets and 45 ml-butanol-1-ol are added. The whole is brought to a boil stirring constantly and butanol extracted with simultaneous saponification of fat. The extraction unit is connected to the reflux condenser and operates for 30 minutes. After complete extraction, 40 ml of sodium phosphate solution are added and homogenized. In the last step, the solution is allowed to stand until separation of the aqueous from the organic phase. 2 μl of the organic phase is detected in the gas chromatograph B-820 (Buchi, Switzerland).

### 3.4.6 Confocal Laser Scanning Microscope (CLSM)

The method of CLSM enables a characterization of the structure. CLSM localized macromolecules such as oil droplets and thus can draw conclusions on the emulsifying and stabilizing properties of egg yolk in emulsion.
Principle:

The CLSM generates virtual sections through an object, which are then assembled into a spatial representation. The laser light source is focused on an x-y-plane at a certain depth of the sample. The laser beam effected at the fluorescent samples the emitting of light of a particular wavelength. In emulsions macromolecules must be selectively stained with fluorescent substances, only then local connections are visible of macromolecules in the food matrix.

CMC solution:

Emulsions have no fixed structure. Therefore, oil droplets are fixed for image setting of screening. This is achieved by the addition of carboxymethyl-cellulose solution (CMC) to the sample. The thickening agent is prepared by insertion of CMC (Merck) in lukewarm water until reached a viscous consistency.

Staining of samples for microscopy:

The emulsion is stained before the measurement with dyes FITC (fluorescein isothiocyanate, Fluka) and Alexa Fluor 555 (Fluka). Subsequently the stained emulsion is immobilized by CMC solution.

Microscopy:

The stained sample is applied to a microscope slide and covered with a coverslip. Microscope slide is analysed with a confocal microscope (Nikon Eclipse C1).

FITC dye diffuses at protein components and Alexa Fluor 555 to phospholipid components. On irradiation by argon laser, the protein molecules emit a green light. The phospholipid components emit red light, if they are irradiated by helium-neon laser. Protein-phospholipid complexes appear light brown on the microscopy images.
4. Results and Discussions

4.1 Pilot-Tests 1

The Pilot-Tests 1 characterized the Premixes of unmodified egg yolk and PLA\textsubscript{2}-modified egg yolk in respect to the changes in viscosity- and stability behaviour in an O / W. (50/50) model emulsion with a protein content of 1.25% as affected by ultra-high pressure homogenization.

4.1.1 Results of Pilot-Tests 1

4.1.1.1 Temperature Development of the Premixes

Taking into account the high increase in temperature in the product (2.4.2) by strong crushing forces during the homogenization process (2.4.1) and the high thermal sensitivity of egg yolk proteins (2.2.5) beside the temperature profile during processing of the Premixes of the unmodified egg yolk and also the thermostable Premixes of PLA\textsubscript{2}-modified egg yolk were investigated.

Figure 20. Effect of homogenizing pressure on the temperature of the Premixes (unmodified and PLA\textsubscript{2}-modified egg yolk) at the exit of the first valve. Initial temperature of the Premix: 5 °C.

Figure 20 shows an identical temperature trend for both Premixes with rising homogenization pressure. The largest delta temperature of 35 °C was generated between 0 MPa and 100 MPa. From the homogenization pressure of 100 MPa, the delta temperature was an average of 22 °C / 100 MPa. The homogenization of the Premix of unmodified egg yolks with 350
MPa resulted in a mixture of hot liquid and vapour and was excluded from the following studies.

4.1.1.2 Rheological Characterization of the Emulsions

Figure 21 and Figure 22 show the results of rheological properties of the model-emulsions prepared from unmodified and PLA2-modified egg yolk. The rheology of descriptive parameters $K$ and $n$ were calculated using the Rheostar 5.0 by Ostwald-de Waele.

![Figure 21: Flow Consistency Index by Ostwald de-Waele of the O/W (50/50) model-emulsions with a protein content of 1.25%, produced with the Premixes of unmodified and PLA2-modified egg yolk which was treated by HPH at different pressures, after 1 day at 7 °C](image)

In Figure 21 is shown the consistency of the O/W (50/50) model-emulsion. The emulsion produced with the untreated (0 MPa) Premix of unmodified egg yolk had a lower consistency than the emulsions with the untreated PLA2-modified egg yolk. The rest of emulsions prepared with treated unmodified egg yolk had all the same comparable consistency (irrespective of the pressure level), which was slightly lower than that of the untreated emulsion. In contrast, the consistency of the emulsions of PLA2-modified egg yolk showed a strong dependence on the level of pressure. The PLA2-emulsion of a 100 MPa homogenized Premix had the largest firmness of all emulsions with a consistency of 1.55 Pa*s$^{-1}$. This firmness was 0.9 Pa * s$^{-1}$ higher than the counterpart of unmodified egg yolk. The PLA2-
Emulsions treated in the pressure range at (150 to 350 MPa) decreased in consistency with increasing homogenization pressure. Starting at a 300 MPa high pressure treatment of the Premixes, the consistency of the emulsion was lower than all other produced emulsions. The emulsion made after treatment at 350 MPa had the lower firmness measured by the Flow Consistency Index which is similar to water.

Figure 22: Flow Behaviour Index by Ostwald de-Waele of the O/W (50/50) model-emulsions with a protein content of 1.25 %, produced with the Premixes of unmodified and PLA2-modified egg yolk which was treated by HPH at different pressures, after 1 day at 7 °C.

Figure 22 shows the shear-thinning (pseudoplastic) behaviour of the O/W (50/50) model-emulsions. The majority of the emulsions had a similar shear-thinning behaviour, except the PLA2-emulsions of 300 and 350 MPa, where the shear-thinning behaviour was reduced with increasing pressure.
4.1.1.3 Macroscopic Stability of the Emulsions

Figure 23 shows the macroscopic appearance of the emulsions at day 1 after production and storage at 25 °C. All emulsions were stable after 1 day and showed no phase separation. All emulsions of unmodified (conventional) egg yolk, as well as the emulsions 0 and 100 MPa from PLA2-modified yolk had localized large air bubbles in the upper part of the emulsion. This effect arose at the production / dispersion of emulsions with the Ultra Turrax.
Figure 24. Stability test of the O/W (50/50) model-emulsions with a protein content of 1.25 %, prepared with the Premixes of unmodified and modified PLA2 egg yolk and treated by HPH at different pressures, after 3 days at 25 °C.

Figure 24 shows the macroscopic appearance of the emulsions at day 3 after production and subsequent storage at 25 °C. All PLA2-emulsions were stable after 3 days and showed no phase separation. All emulsions of unmodified (conventional) egg yolk, as well as the emulsions of 0 and 100 MPa from PLA2-modified yolk were not able to stabilize the large air bubbles in the upper part of the emulsion and thus a loss of total volume occurred. The same effect was observed in the emulsions PLA2 (150 to 300 MPa) but to a lesser extent. All emulsions of unmodified egg yolk showed the effect of creaming in which water settles in the lower part of the emulsion. Furthermore, all of these emulsions were unstable after 3 days, due to phase separation. The separated oil was visible in the upper part of the emulsion. The emulsions of thermostable (PLA2) egg yolk (except 250 to 350 MPa) showed the effect of creaming. The creaming was significantly less pronounced than in the emulsions of conventional egg yolk.
Figure 25. Stability test of the O/W (50/50) model-emulsions with a protein content of 1.25%, prepared with the Premixes of unmodified and modified PLA$_2$ egg yolk and treated by HPH at different pressures, after 7 days at 25 °C.

Figure 25 shows the macroscopic appearance of the emulsions at day 7 after production and storage at 25 °C. The emulsions showed no progress in the creaming. However, the phase separation of emulsions of untreated egg yolk markedly increased after 7 days. Likewise it turned out, that all emulsions of themostable egg yolk were unstable after 7 days, due to the phase separation. The best performance (over the entire duration of the stability tests) was the PLA$_2$-emulsion of 350 MPa, because they did not show any loss in volume and no creaming.
4.1.2 Discussion of Pilot-Tests 1

The temperature increases in the two types of Premixes were between 20-23 °C / 100 MPa, and thus just over the results of Picart et.al. [2006] who described an increase of 15-20 °C / 100 MPa. The identical temperature development in both types of Premixes for the same pressure range is based on the similarity of egg yolk matrices (2.3.3). Nevertheless, the hydrolysis of phospholipids to lysophospholipids makes Premix of PLA₂-modified egg yolk stable to the temperature of 96 °C (350 MPa homogenizing pressure). At this high temperature, phosvitin (2.2.5) is the only phospholipid which is stable of the conventional (unmodified) egg yolk. Phosvitin is not able to stabilize in the structure of proteins, because of its low content naturally present in the egg yolk (Figure 5) of 4% (2% in Premix). However, the structure of the PLA₂-modified egg yolk is protected from the entire unfolding of proteins by the complex formation (2.3.3) of Apo-LDL to the lysophospholipids and free fatty acids.

Usually egg yolk proteins start to denature at a temperature between 65 and 70 °C. The fact that the structure of the Premix of unmodified egg yolk is not completely denatured in the pressure range 250 to 300 MPa (75 - 85 °C; Figure 2.0) was due to the short duration of maximum temperature (also minimized by the external cooling). Only at the homogenization pressure of 350 MPa proteins and its structure denatured completely. The Premixes of PLA₂-modified egg yolk were apparently more resistant to high temperatures / high pressure. Nevertheless, the rheology of the PLA₂-emulsion showed that the firmness of the emulsion decreases continuously from a pressure of 250 MPa. However, the homogenization treatment at lower pressure (i. e. 100 to 250 MPa) unfolded proteins in the Premix to a degree, so that the stabilising behaviour increased significantly in comparison to the untreated (0 MPa) PLA₂-Premix. It should be mentioned, the results by Dutilh and Groger [1981] (in 2.3.3) confirmed in the Pilot-tests 1. Due to that the untreated Premix of PLA₂-egg yolk provided to the emulsion an increasing consistency in contrast to the untreated premix of unmodified egg yolk. It can further be mentioned that the high-pressure homogenization of conventional (unmodified) egg yolk has a negative effect on the emulsion, because it reduced the consistency, regardless of the pressure level of homogenization.

The stability of the emulsions of PLA₂-modified egg yolk was significantly better than that of the emulsions prepared with conventional egg yolk. The emulsions of unmodified egg yolk were already unstable on the day 3 after production during whereas the PLA₂-emulsions were unstable before the day 7 after production. Furthermore, the results confirm the statements by Heinzelmann et al. [1994] (in 2.5) with respect to the foaming ability of high-pressure homogenized egg white proteins. It should be mentioned in this context that the industrial separation of egg yolk and egg white is never completed. A fraction of 15% egg white is not uncommon in the liquid egg yolk. At this point, this relationship which will result in
an air bubbles stabilization and a better stability in the treated PLA₂-modified emulsions cannot be assured but it might be a reasonable justification of the experimental data.

In summary, it can be said that the high-pressure homogenized Premixes of enzyme modified egg yolk showed a better performance with respect to the viscosity and the stability of the emulsions. Therefore, in the Pilot-Tests 2 only is used the PLA₂-modified egg yolk. In addition, the range of homogenizing pressures is reduced to 0 (untreated), 200, 225, 250, 300 MPa based on the results. 350 MPa is discarded for further experiments due to the very low viscosity of the emulsion prepared using this egg yolks and the very high material stress in the fault-prone ultra-high-pressure homogenizer (*FPG11300 Hygenic Homogeniser*). The pressure level of 300 MPa can be also simulating a high stress in the proteins. Despite the rheological properties of the emulsions obtained with processing pressures of 100 and 150 MPa were very good, they are eliminated for further trial because of its slight creaming in the emulsion with PLA₂-modified egg yolk and because the other homogenization pressures generated better stabilities. The homogenization pressure of 225 MPa is incorporated to the Pilot-Tests 2 as an interesting and potential pressure likely allow the generation a good balance between viscosity and stability in emulsion.

### 4.2 Pilot-Tests 2

The Pilot-Tests 2 examines the influence of different ratios (recipes) of oil to water (i.e.10/90; 35/65 and 50/50) and different amounts of ultra-high pressure homogenized PLA₂-modified egg yolk (i.e. protein content: 1.25% and 1.625%). Furthermore, the emulsions were re-examined after 10 days, to determine whether the high-pressure homogenised yolk shows a different behaviour than the untreated egg yolk.

The aim is to find the recipe which produces the emulsion with highest viscosity and stability. Based on the influence of high-pressure homogenized egg yolk on the stability- and emulsifying capacities in the emulsion, a recipe will be selected for the subsequent Main-Tests.
4.2.1 Results of Pilot-Tests 2

4.2.1.1 Rheological Characteristics of Emulsions

In Figure 26 to Figure 28 and Table 4 are illustrated the results of the analysis of the rheological properties of the model-emulsions. The model-emulsions were stored at 7 °C and analysed on day 1 and 10 after production. The rheology of descriptive parameters $K$ and $n$ were calculated using the Rheostar 5.0 by Ostwald-de Waele.

![Figure 26](image-url)

**Figure 26.** Flow Consistency Index by Ostwald de-Waele of O/W (10/90) model-emulsion with different protein content (1.25% and 1.625%), produced with the Premixes of PLA$_{25}$ modified egg yolk which was treated by HPH at different pressures, after 1 and 10 days at 7 °C.

The O/W (10/90) emulsions of Figure 26 had low consistency. The flow consistency index was located in a small range of about 0.005 to 0.025 Pa*s$^{-1}$. The flow behaviour index from Table 4 (shown in chapter 8.) yielded for all 10/90 emulsions a value of approximately 1. This means that the samples did no showed the typical pseudoplastic (shear-thinning) behaviour of an emulsion, but a Newtonian behaviour (such as water). There is no further description of the individual values of the emulsions from Figure 26 and the related flow behaviour index, due to the small differences in their rheological properties and the Newtonian behaviour.
The O/W (35/65) emulsions of Figure 27 had a 10-times stronger consistency with about 0.07 to 0.3 Pa *s$^{-1}$ than the O/W (10/90) emulsions. Flow behaviour Index (Table 4) with the values from 0.55 to 0.77 was also smaller, and showed typical pseudoplastic (shear thinning) behaviour of emulsion. The emulsions having a protein content of 1.625% had a higher overall firmness than the emulsions with a protein content of 1.25%. The exception was the emulsion (protein content: 1.625%) with the 300 MPa treated Premix, which had a comparable firmness like its counterpart with a protein content of 1.25%. The highest viscosity (0.292 Pa*s$^{-1}$) had the emulsion (protein content: 1.625%) with the Premix treated at 200 MPa. To put it in a nutshell, the high-pressure homogenization of egg yolk was found to result in emulsion of higher viscosity as compared to the emulsion of untreated egg yolk, except the already mentioned 300 MPa emulsion (protein content: 1.625%).

The emulsions lost the firmness after 10 days of storage at 7 °C. The loss in viscosity was highest in the emulsions with a protein content of 1.625% as the viscosity at day 1 was also much higher compared to the emulsions with 1.25 % of protein content. This makes that both type of emulsions with different protein contents has comparable at day 10.
Figure 28. Flow Consistency Index by Ostwald de-Waele of O/W (50/50) model-emulsion with different protein content (1.25% and 1.625%), produced with the Premixes of PLA2-modified egg yolk which was treated by HPH at different pressures, after 1 and 10 days at 7 °C.

The O/W (50/50) emulsions of Figure 28 had a 10-times stronger consistency with about 0.5 to 2.3 Pa s⁻¹ than the O/W (35/65) emulsions. Flow behaviour Index (Table 4) with the values from 0.55 to 0.77 had similar shear-thinning behaviour as the O/W (35/65) emulsions. The emulsions having a protein content of 1.625% had a higher firmness than the emulsions with a protein content of 1.25%. The exception was the emulsion (protein content: 1.625%) with the untreated Premix, which has lower consistency than the emulsions (protein content: 1.25%) out of the 200, 225 and 250 MPa treated Premixes. The highest viscosity (2.3 Pa s⁻¹) had the emulsion (protein content: 1.625%) with the 250 MPa treated Premix. Furthermore, the high-pressure homogenization of egg yolk turned out in higher viscosity compared to the emulsions of untreated egg yolk (0 MPa control samples), except the emulsion 300 MPa (protein content: 1.25%).

The emulsions lost the firmness after 10 days of storage at 7 °C. The loss in viscosity was highest in the emulsions with a protein content of 1.625%, so that the firmness of all high-pressure homogenized emulsions was comparable with the emulsion (protein content: 1.25%) of the untreated egg yolk at day 1.
4.2.1.2 Droplet Size Distribution and Surface to Volume Ratio

In Figure 29 to Figure 34 and Table 5 are shown the results of the analysis of oil droplet size distribution and the surface to volume ratio (specific surface area) of the oil droplets in model-emulsions. The model-emulsions were stored at 7 °C and analysed on day 1 and 10 after production. The descriptive parameters are sauter mean diameter, dispersion index (SPAN), specific surface area and droplet size distribution curve.

Figure 29. Effect of oil concentration (10, 35 and 50%) and HPH level (200, 225, 250, 300 MPa) on the sauter mean diameter of O/W emulsions with protein content (1.25 and 1.625 %), after 1 day at 7 °C.

Figure 29 shows the influence of oil concentration, protein content and high-pressure homogenization level of modified egg yolk on the sauter mean diameter of the oil droplets in emulsion. The sauter mean diameter was hardly affected by the oil concentration in the emulsion. Higher concentration of proteins in the emulsion reduced sauter mean diameter. The high pressure homogenization of egg yolk slightly increased the sauter mean diameter compared to untreated (0 MPa) egg yolk. The highest value for the emulsion series achieved with the pressure of 300 MPa and an oil concentration ex 35% in the emulsion.
Figure 30 shows the influence of oil concentration, protein content and high-pressure homogenization level of modified egg yolk on the dispersion index of the oil droplets in emulsion. A decrease in the dispersion index was observed with increasing concentration of oil in the emulsion. The combination of high protein concentration (1.625%) and a low concentration of oil (O/W: 10/90) in the emulsion led to higher dispersion index, compared to the other emulsions series. The emulsions series having a protein concentration of 1.625% had a slightly higher dispersion index than the emulsions series having a protein concentration of 1.25% with the same content of oil. The high pressure homogenization of egg yolk showed similar values of dispersion index, for equal oil and protein concentration in emulsions.
Figure 31. Effect of oil concentration (10, 35 and 50%) and HPH level (200, 225, 250, 300 MPa) on the specific surface area of O/W emulsions with protein content (1.25 and 1.625 %), after 1 day at 7 °C.

Figure 31 shows the influence of oil concentration, protein content and high-pressure homogenization level of modified egg yolk on the specific surface area of the oil droplets in emulsion. The specific surface area was hardly affected by the oil concentration in the emulsion. The emulsions with an oil concentration ex 35% had a continuous decrease in the specific surface area of the oil drops with increasing homogenization pressure. Higher protein content (1.625%) in the emulsions increased the specific surface area of the oil droplets in comparison to the emulsions with lower protein content (1.25%), for equal concentrations of oil in in emulsions.
Figure 32. Effect of oil concentration (10, 35 and 50%) and protein content (1.25 and 1.625 %) on the oil droplet size distribution of O/W emulsions, after 1 day at 7 °C.

Figure 32 shows the influence of oil concentration and protein content on the oil droplet distribution in emulsion. With increasing oil concentration the oil droplet size distribution changed from a monomodal towards a bimodal system. The proportion of small oil droplets in an emulsion was increased by higher protein content in the emulsion.

Figure 33. Effect of HPH treatment of protein content (1.25 %) on the oil droplet size distribution of O/W (50/50) emulsions, after 1 day at 7 °C.

Figure 33 shows exemplarily for all emulsions, the influence of high pressure homogenized egg yolk on the oil droplet size distribution in the O/W (50/50) emulsion with a protein content of 1.25%. The high pressure homogenization of egg yolk hardly influences the droplet size distribution of the further prepared emulsions. Furthermore, the emulsions at day 10 (Figure
34) showed no changes in the oil droplet distribution compared to the emulsions at day 1 (of Figure 33).

Figure 34. Effect of HPH treatment of protein content (1.25 %) in O/W (50/50) on the oil droplet size distribution of O/W (50/50) emulsions, after 10 days at 7 °C.

Table 5 (chapter 8) and the exemplary Figure 33 and Figure 34 showed the emulsions had only minor changes after 10 days of production on the particle size distribution and specific surface area of the oil droplets, as compared to the samples analysed from day 1.
4.2.1.3 Macroscopic Stability of Emulsions

Figure 35. Effect of oil concentration (10, 35 and 50%) and HPH (200, 225, 250, 300 MPa) treated protein content (1.25 and 1.625 %) in O/W emulsions on its stability, after 14 days at 7 °C.

Figure 35 shows the macroscopic appearance of all emulsions after 14 days of production and storage at 7 °C. It can be observed that the creaming in the emulsions decreased with increasing concentration of oil. Higher protein content, 1.625%, in the emulsions decreased only slightly the effect on the creaming. The influence of high pressure homogenized egg yolk on the creaming was only visible at the highest pressure of 300 MPa emulsions. This pressure level decreased the creaming a little bit in emulsion compared to the other pressures applied in the study at the same concentration of oil and protein. The exception
was the O/W (50/50) emulsion of a protein content of 1.625% at 225 MPa. The creaming effect was comparable with the emulsion of same recipe at 300 MPa.

In the O/W (50/50) emulsions were appeared again air bubbles like in the Pilot-Tests 1. These air bubbles became larger during the stability test. The O/W (50/50) emulsion of a protein content of 1.25% showed a greatly reduced volume after 14 days, especially the untreated and 300 MPa egg yolk emulsion. Likewise, the emulsion showed a white diffuse continuous phase (lower part of the emulsion).

All emulsions were stable as they showed no phase separation of oil on the emulsion surfaces.

4.2.2 Discussion of Pilot-Tests 2

It is noted, the biggest influencing factor on the viscosity (see Tesch [2002] in 2.1.2), and stability of an emulsion is the concentration of oil in the recipe. The protein content in the emulsion and the previous high-pressure homogenization of egg yolk had a much lower influence. The influence of high pressure treated egg yolk on the rheological properties increased with increasing amount of oil in the emulsion. The high-pressure-treated egg yolk obtained similar emulsifying capabilities in an emulsion to the untreated egg yolk with a larger percentage in the emulsion. Concrete terms, the untreated O/W (50/50) emulsion having a protein content of 1.625% showed the same or even lower rheological properties than the emulsions with a high-pressure homogenised (200, 225 and 250 MPa) and protein content of 1.25%. Hence, the statements of Floury et.al. [2002] (in 2.5) have been confirmed with the results obtained in the Figure 26 to Figure 28 and Table 4. The consistency and pseudoplastic (shear thinning) behaviour of emulsions with high pressure homogenised egg yolk was greater than that of the emulsion to untreated egg yolk. The 300 MPa high-pressure homogenised egg yolk caused a strong fluctuation in the values of the consistency. More precisely, in some emulsions the viscosity of the emulsions of 300 MPa was similar to a low high-pressure homogenised egg yolk and in other emulsions the consistency value was lower than the emulsion of untreated egg yolk. Two causes can be responsible for this phenomenon. Firstly, 300 MPa is in the range, where the egg yolk is denatured to such an extent, that it decreased the stabilization of the aqueous phase. Secondly, at this higher pressure the homogenizer operates with pressure fluctuations (up to 20 MPa) at the Premix production, so that some of Premixes (proteins) were treated more strongly than other Premixes. The exact reasons influencing protein denaturation during high-pressure
homogenization and thus protein functionality such as crushing forces, temperature and protein denaturation are explained in the chapters 2.3.3, 2.4.1, 2.4.2 and 4.1.2.

The different concentration of oil in the emulsion did not affect sauter mean diameter and specific surface area. The dispersion index (SPAN) was influenced by the concentration of oil in the emulsion. Higher concentration of oil in the emulsion led to smaller width in the droplet size distribution. Furthermore, the oil concentration affected the characteristics of the oil droplet size distribution, with increasing concentration of oil in an emulsion shifted the droplet size distribution from monomodal to bimodal. This observation could be explained by the dispersion process during the preparation of the emulsion. Rotor stator systems (Ultra Turrax) create wide and uniform crushing zone with an unfavourable distribution of a low energy input (Anbarci [1987] in 2.4.1). This effect intensifies with increasing concentration of oil and an unchanged input of energy in an emulsion and leads to a bimodal droplet size distribution. In emulsions an increase in the protein content, from 1.25 to 1.65% result in a shift in the distribution curve to a bimodal droplet size distribution curve (Figure 32). This means, the fraction of smaller droplets was increased in the emulsion, due to higher concentration of emulsifier which better stabilized the smaller droplets after its formation (2.1.5).

Figure 29 to Figure 34 and Table 5 confirm the results from Floury et.al. [2002], high pressure homogenization of Premixes of egg yolk had little effect on the size distribution and specific surface area of oil droplets in emulsions.

The repeated analysis of the emulsions after 10 days (shown in Table 5) showed no major differences in the oil droplet size distribution and the specific surface area compared to the results after day 1. This means that the emulsion interfacial films of all analysed emulsions were stable and there was no coalescence and / or Ostwald-ripening on a larger scale. Coalescence and Ostwald-ripening (see 2.1.3) would have been resulted in an increase in the sauter mean diameter and a decrease of the specific surface area. As a result, the creaming is based solely on oil concentration and protein content in the emulsion. The creaming is reduced by an increase in oil concentration due to more densely packed oil droplets. The individual droplets can accelerate to a lesser extend until they reach the next oil droplet. The viscosity in the aqueous phase was also increased by higher protein content (mentioned in 2.1.6). This also slows down the speed of movement of the oil droplets in the aqueous phase and thus reduces the creaming. High-pressure homogenization of egg yolk had only an influence on the stability (creaming) of the emulsion at a high pressure. However, precise statements can only be based on the CLSM images (see 4.3.2.6) made in the Main-Tests study phase.
The stability tests in Figure 35 showed no phase separation and thus all emulsions were stable after 14 days. The influencing factors of creaming already mentioned. It should be noted that inclusion of air bubbles (O/W (50/50) emulsion) depends on the concentration of oil. However, the stabilization of air bubbles depends on the protein content and level of homogenizing pressure. The volume loss of the emulsions with a protein content of 1.25%, correlated with the decreased stabilization of air bubbles. Whereas the air bubbles can be stabilized better with higher homogenization pressure and protein content (1.625%), which in turn correlates with the already mentioned protein folding and discussion of 4.1.2 (see Heinzelmann et al. [1994]).

In the specific case of the O/W (50/50) emulsion with a (300 MPa treated hydrolysed egg yolk) protein content of 1.25% showed the influence of an excessive pressure on the properties of emulsions such as viscosity and stability. The white continuous phase (4.2.1.3) showed completely denatured proteins which was dissolved in the aqueous phase due their loss of structure. This resulted in a lower number of stabilizing proteins in the continuous phase and at the interface of the oil droplets. The lower number of stabilizing proteins in the continuous phase led to a reduction of consistency of emulsion (Figure 28). The lower number of stabilizing proteins at the interface led to a reduction of stability in emulsion (Figure 35) such as loss of volume (air bubbles) and increase of creaming.

The O/W emulsion with a protein content of 1.625% demonstrated the best performance of all analysed emulsions, with respect to the rheological properties, the stability and the specific surface area. For this reason for the next experimental phase, Main Test, the protein content of the emulsion is increased to 2% to make sure the protein requirement to prepare an stable emulsion is correct. The concentration of oil in the emulsion remains the same.

4.3 Main-Tests

Based on the results of the Pilot-Tests 2 exclusively the high-pressure homogenized levels, 225 and 300 MPa, and enzyme modified- PL\(_2\) egg yolk were used in the Main-Tests. The emulsions from untreated conventional and untreated enzyme modified-PLA\(_2\) egg yolk variants as control samples. The main focus of the investigations was to induce a change in structure of egg yolk by the ultra-high pressure homogenization and investigate its effect on the microstructure of the emulsion. These structural changes will help to understand the influence of egg yolk with respect to emulsifying, stabilizing and rheological behaviour in the emulsion.
4.3.1 Results of Egg Yolk

4.3.1.1 Analysis of Dynamic Interfacial Tension

Figure 36. Dynamic Interfacial Tension between Miglyol and diluted Premix (1/100 w/w; 0.08% protein content) of PLA₂-modified or non-modified and treated or untreated egg yolk as a function of the droplet formation time.

The four types of egg yolk studied showed no differences concerning reduction of the dynamic interfacial tension of Miglyol (oil) with increasing droplet formation time (Figure 36).
4.3.1.2 Investigations of Egg Yolk Structure by SEM-Images

The freezing of the Premix of egg yolk and subsequent sublimation of water made visible the protein structures which are shown in Figure 37.

![Figure 37. SEM-images of Premixes (diluted egg yolk; 50/50 w/w). a: unmodified and untreated egg yolk; b: PLA\(_2\)-modified and untreated egg yolk; c: PLA\(_2\)-modified and 225 MPa treated egg yolk; d: PLA\(_2\)-modified and 300 MPa treated egg yolk (2% protein content); at 2500 magnification.](image)

The Scanning Electron Microscopy (SEM) images showed no differences in structure between the unmodified untreated egg yolk (Figure 37a) and the enzyme-modified untreated egg yolk (Figure 37b). The comparison between the already mentioned SEM images and the PLA\(_2\)-modified egg yolk processed by high-pressure homogenization (at 225 MPa: Figure 37c) revealed a thickening of the network structure of protein. The network also showed fewer cavities likely due to cross-links and was therefore denser. The protein network of the PLA\(_2\)-modified egg yolk and processed high pressure homogenized at 300 MPa (Figure 37d) had even more cross-links than the structure of the 225 MPa egg yolks. The structure of the image has a more rounded structure in contrast to the linear structures of the other images (a, b, c).
4.3.2 Results of Emulsion

4.3.2.1 Rheological Characteristics of Emulsions

The rheology was calculated using the Rheostar 5.0 by Ostwald-de Waele. The results from Figure 38 will specify the flow consistency index $K$ and flow behaviour index $n$.

![Figure 38. Flow consistency index [K] and Flow Behaviour Index [n] by Ostwald de-Waele of the O/W (50/50) emulsions with PLAs-modified or unmodified and treated or untreated egg yolk (2% protein content).](image)

The viscosity of the O/W (50/50) emulsion with unmodified egg yolk (unmodified_0 MPa) was considerably lower than the consistency of enzyme-modified emulsion (PLA2_0 MPa). The high-pressure homogenization of hydrolysed egg yolk at 225 MPa increased the consistency again. A further increase of the homogenizing pressure in enzyme-modified egg yolk (to 300 MPa) reduced the consistency of the emulsion just below that of the untreated emulsion (PLA2_0 MPa). The emulsions did not differ in their shear thinning behaviour.
4.3.2.2 Droplet Size Distribution and Surface to Volume Ratio

Figure 39. Oil droplet size distributions and specific surface area of the O/W (50/50) emulsions with PLA\textsubscript{2}-modified or unmodified and treated or untreated egg yolk (2\% protein content).

The descriptive parameters are sauter mean diameter, dispersion index (SPAN) and specific surface area. Figure 39 shows no differences in the emulsions in the mentioned parameters in comparison to the values from the Figure 29 to Figure 31. The emulsions did not differ to each other, excluding the emulsion processed at 300 MPa. This emulsion had a slightly larger oil droplets and wider distribution of them. Therefore, the specific surface area was slightly decreased.
Figure 40. Stability of the O/W (50/50) emulsions with different types of egg yolk (2% protein content). UM: unmodified and untreated egg yolk; 0: PLA₂-modified and untreated egg yolk; 225: PLA₂-modified and 225 MPa treated egg yolk; 300: PLA₂-modified and 300 MPa treated egg yolk; at day 1 / 3 / 7 / 10 after production.

Figure 40 shows the macroscopic appearance of the emulsions at day 1, 3, 7 and 10 after production and storage at 7 °C. The emulsion made of unmodified egg yolk showed at day 1 after production the effect of creaming. All the other emulsions made of enzyme-modified egg yolk were stable with respect to the creaming. These emulsions showed a very differently creaming on the third day after production. With increasing pressure (0 to 300 MPa; PLA₂) at egg yolk, the creaming decreased in the emulsion. The emulsion prepared with unmodified egg yolk had a continuous increase of creaming in the emulsion for the duration of the stability test. The same applies to emulsions with the enzyme-modified egg yolk, but to a lesser extent.
Figure 41. Stability of the O/W (50/50) emulsions with different types of egg yolk (2% protein content). UM: unmodified and untreated egg yolk; 0: PLA$_2$-modified and untreated egg yolk; 225: PLA$_2$-modified and 225 MPa treated egg yolk; 300: PLA$_2$-modified and 300 MPa treated egg yolk; at day 14 after production.

Figure 41 shows the macroscopic appearance of the emulsions at the end of the stability test (14 days after production and storage at 7 °C). The emulsions made of enzyme-modified egg yolk had a lower creaming than the emulsion made of unmodified egg yolk. The emulsion with the greatest homogenizing pressure on egg yolk was most stable one against the creaming. The creaming increased at smaller homogenization pressures of egg yolk. The emulsions prepared with untreated egg yolk had large air bubbles in the upper part of the emulsion. However, the emulsions with high-pressure homogenised egg yolk showed a fine air bubble distribution over the entire emulsion (like in 4.2.1.3).

All emulsions showed no phase separation during the stability tests and were therefore stable.
4.3.2.4 Protein-Interfaces-Occupancy of Emulsions

![Protein-Interfaces-Occupancy of Emulsions](image)

Figure 42. Protein-Interfaces-Occupancy of the O/W (50/50) emulsions with PLA₂-modified or unmodified and treated or untreated egg yolk (2% protein content).

The protein interfaces occupancy in Figure 42 was calculated by the analysis of the protein amount in the dispersed phase of the emulsion. The emulsion with untreated, thermostable egg yolk (PLA₂_0 MPa) had with 6 mg proteins per square meter of oil droplet surfaces, the smallest interfaces occupancy. Protein interfaces occupancy 7 mg m⁻² was slightly greater in the emulsion with unmodified egg yolk (unmodified_0 MPa). The high-pressure homogenization of egg yolk (PLA₂_225 MPa and 300 MPa) caused an increase of the protein interfaces-occupancy in the emulsion with increasing pressure.

4.3.2.5 Extractable Fat Fraction (EFF) of Emulsions

EFF specifies how much fat (oil) was extracted of the disperse phase of the emulsion and thus it is a measure of the stability of the interface film of the oil droplets and the stability of the entire emulsion.
Figure 43. Extractable Fat Fraction (EFF) of the O/W (50/50) emulsions with PLA₂-modified or unmodified and treated or untreated egg yolk (2% protein content).

The highest proportion of fat (oil) could be extracted (4.23%) from the untreated emulsion with unmodified egg yolk (unmodified_0 MPa). The emulsion with 300 MPa treated egg yolk (PLA₂_0 MPa) yielded only about 2.08% extractable fat. The other two emulsions with enzyme-modified egg yolk (PLA₂_0 MPa and 225 MPa) had the lowest fat extraction (approx. 1.34%).

4.3.2.6 Investigations of Emulsion by CLSM-Images

CLSM images enable an assessment of the microstructure and the structure of the interface of emulsions. The interior of the oil droplets appears black in the pictures. The selective staining (see 3.4.6) with a fluorescent material leads to dyed red phospholipids, green coloured proteins and light brown stained protein-phospholipid complexes. The CLSM images on the left side (10-fold magnifications) of Figure 44 to Figure 47 show only the microstructure of the emulsions. The images on the right side (100-fold magnifications) of the Figure 44 to Figure 47 show the detailed microstructure and the structure of the oil interface of the emulsion. The enlarged section of the right images shows the detailed structure of the interface film of oil droplets.
The left image of Figure 44 shows an inhomogeneous microstructure of the O/W (50/50) emulsion with a protein content of 2% from unmodified untreated egg yolk. It can be observed four very large oil droplets (diameter about 90 μm) with large accumulations of phospholipids and protein-phospholipid complexes. Furthermore, the inhomogeneous distribution of the dispersed phase was identified by the large dark green areas, which is the continuous phase (without oil droplets). The 100-fold magnification showed a variety of large protein aggregates covered by phospholipids. These aggregates are located in the continuous phase and at the interfaces of the oil droplets. In addition, these protein aggregates connect the oil droplets to flocs. The enlarged section of the right image shows oil droplets with a thin interfacial film of protein-phospholipid complexes and protein aggregates mostly covered by phospholipids.
Figure 45. CLSM images of the oil droplet interface of the O/W (50/50) emulsions prepared with PLA₂-modified and untreated egg yolk (2% protein content) at different image magnifications (left. pic. 10x; right. pic. 100x).

The Figure 45 shows the microstructure and interface structure of the O/W (50/50) emulsion having a protein content of 2% of modified untreated egg yolk. This emulsion had a homogeneous microstructure as no large areas of continuous phase can be seen (image with a 10-fold magnification). The right image with a 100-fold magnification showed densely packed oil droplets. Very few small protein aggregates are visible with phospholipid covering. In contrast to the emulsion of unmodified untreated egg yolk, the proportion of protein-lysophospholipid complexes (see 2.3.3) increased in the vicinity of the oil droplets. The enlarged section shows three contiguous oil droplets having thick homogeneous interfacial films of protein-lysophospholipid complexes.

Figure 46. CLSM images of the oil droplet interface of the O/W (50/50) emulsions prepared with PLA₂-modified and by 225 MPa treated egg yolk (2% protein content) at different image magnifications (left pic. 10x; right pic. 100x).
The Figure 46 shows the microstructure and interface structure of the O/W (50/50) emulsion having a protein content of 2% of modified and high-pressure homogenised at 225 MPa egg yolk. This emulsion showed less homogeneous microstructure (in comparison to Figure 45) as the distribution (density) of the oil droplets was irregularly. The right image with a 100-fold magnification shows a high density of contiguous oil droplets. The very small protein aggregates with phospholipid casings appeared very limited. The red distorted spots might be phospholipids / lysophospholipids which have linked to the protein-lysophospholipid complexes. It seems that every droplet of oil was linked via the protein-lysophospholipid complexes and phospholipids / lysophospholipids directly to the surrounding oil droplets. The enlarged section shows a very thick homogeneous interfacial films of protein-lysophospholipid complexes, which protruded partially into the continuous phase or linked the interfacial films of the immediately surrounding oil drops. This is a protein network with flocs.

![Figure 46](image)

**Figure 46.** CLSM images of the oil droplet interface of the O/W (50/50) emulsions prepared with PLA₂-modified and by 300 MPa treated egg yolk (2% protein content) at different image magnifications (left pic. 10x; right pic. 100x).

The Figure 47 shows the microstructure and interface structure of the O/W (50/50) emulsion having a protein content of 2% of modified 300 MPa high-pressure homogenised egg yolk. This emulsion had an inhomogeneous microstructure, because the dispersed phase was densely packed all over the picture (Figure 47, left image). In other places there were large areas of continuous phase. The right image with a 100-fold magnification was similar to the right image of Figure 31. The only difference was the network of protein-lysophospholipid complexes, which showed less linkage of oil droplets. The enlarged section shows very irregularly thick interfacial films of protein-lysophospholipid complexes and a few small aggregates of proteins and lysophospholipids / phospholipids.

![Figure 47](image)
4.3.3 Discussion of Main-Tests

4.3.3.1 Influence of Ultra-High Pressure Homogenization (UHPH) Processing in Egg Yolk

The scanning electron microscopy (SEM) shows the effect of high pressure homogenization on the protein structure of the egg yolk. This allows a better understanding of the emulsifying and stabilizing behaviour in an emulsion. The analysis of the SEM images (4.3.1.2) confirmed the hypothesis from the discussion of the Pilot-Tests 1 (4.1.2) and the statement (2.3.3) regarding to the similarity of both egg yolk matrixes. The hydrolysis of egg yolk with PLA2-enzymes caused no visible change in the network structure of protein compared to conventional (unmodified) egg yolk. The turbulent flows of high pressure homogenization and the resulting disruption forces (2.4.1) as shearing, inertia, cavitation, high-speed impacts and shock waves had substantial influence on the nature of the structure of the network and its proteins. The same applies for the high temperatures (2.4.2 and 4.1.1.1), which are generated from the disruption forces. This high level of product stress led to a partial denaturation and unfolding of proteins. This was reflected in the results observed in the SEM-images. The high-pressure homogenized egg yolk (PLA2_modified) at 225 MPa had significantly more distinctive networks and thickened protein threads. The homogenization pressure of 300 MPa induced in the thermostable egg yolk a further strengthening of the network by protein threads that enables no longer an identification of individual protein threads. Due to the formation of the complex of apo-LDL, lysophospholipids and free fatty acids the folding of the enzyme-modified proteins was not complete, although the denaturation was not prevented. Nevertheless, this formation of this complex prevents the destruction of proteins and its networks (Mine, 1997; Daimer and Kulozik, 2008; refer to 2.3.3]. The investigation of Marco-Moles et al. [2012] (2.5) describes what happens to non-thermostable protein by the high-pressure homogenization. Egg yolk and milk proteins denatured very strongly at a homogenization pressure of 250 MPa and form aggregates on its self, which leads in an immediate phase separation in the emulsion. For this reason, the different degrees of cross-linking of proteins (4.3.1.2) provide the following discussion still a valuable contribution, with respect to the high-pressure homogenization of egg yolk and its effect on the characteristics of the emulsions.
4.3.3.2 Influence of UHPH-Processing of Egg Yolk on the Microstructure and the Interfacial Film of the Emulsion.

The Confocal Laser Scanning Microscope (CLSM) provides a view of the microstructure and the interfaces of emulsions. Therefore, CLSM images (4.3.2.6) enable an explanation for the effect of the enzyme modification and high-pressure treatment of egg yolk to the characteristics (e.g. stability, rheology) of an emulsion.

The conventional pasteurized, homogenized (unmodified and untreated) egg yolk as an emulsifier in an O/W (50/50) emulsion with 2% protein content (Figure 44) had a very inhomogeneous structure. The inhomogeneous structure was marked by large areas of continuous phase and the droplet aggregation (2.1.3) such as flocculation and coalescence / Ostwald-ripening. Coalescence and Ostwald-ripening were visible to the very large oil drops (about 90 μm in diameter). The confluence of several droplets to a large drop was developed by the unstable interfacial films of the oil droplets (2.1.3). In this case, the very thin interfacial films were not sufficiently stabilized by protein-phospholipid complexes. The reasons for the very thin interfacial films were the aggregated protein and -clusters. Because of the low solubility in the continuous phase and the lack of flexibility to stabilize the interface of the oil droplet the majority of proteins (mainly granule) were not involved in the stabilization of the interface.

The untreated thermostable (PLA₂ modification) egg yolk was completely the opposite to conventional egg yolk. It gave the emulsion (Figure 45) a homogeneous structure, due to a thicker and homogeneous interfacial film by the protein-lysophospholipid complexes. The proportion of protein aggregates in the emulsion decreased drastically due to the enzyme modification of egg yolk. This is explained by the partial rupture of the granules and thus the increases in the solubility of the proteins (Daimer and Kulozik [2009] in 2.3.3). The modification of the egg yolk by the PLA₂-enzyme did not prevent flocculation of oil droplets in the emulsion. However, signs of other destabilizing mechanisms such as Ostwald-ripening or coalescence were not detectable.

The high-pressure homogenization at 225 MPa of the enzyme modified egg yolk altered the emulsion (Figure 46) on some points of the emulsion compared to the untreated PLA₂-modified egg yolk. First, the structure of the emulsion was no longer homogeneous. Secondly, the interfacial films were slightly thicker and the protein-lysophospholipid complexes protruded partially in the continuous phase. Thirdly, the other protein-lysophospholipid complexes form a dense network between the oil droplets. This network formation in the emulsion was confirmed by the SEM images of 4.3.1.2 and accordingly to the discussion 4.3.3.1. This implies a stronger network formation of the protein threads in the
egg yolk with increasing of the homogenization pressure. Further increase of the homogenizing pressure (300 MPa) on the egg yolk reduced the network between the oil droplets in the emulsion (Figure 47). In relation to the discussion 4.3.3.1, the denaturation of the egg yolk was too high. The cross-linked protein threads were partially destroyed in the preparation (dispersion) of the emulsion by the occurring shear forces. And a complete new cross-linking was no longer possible in the emulsion. The now increased number of small protein aggregates at the interface is an indication of a less dense network, because of the proteins of the aggregates were no longer available for the network formation of protein-lysophospholipid complexes. This is reflected in a much greater inhomogeneity of the emulsion (similar to the conventional emulsion of egg yolk). The interfacial films of the oil droplets were inhomogeneously thickly covered with/of protein-lysophospholipid complexes and a few proteins and phospholipids.

None of the tested emulsions was stable against flocculation of oil droplets.

4.3.3.3 Influence of UHPH-Processing on the Emulsifying Behaviour of Egg yolk in an Emulsion

The enzyme modification and high-pressure homogenization of egg yolk had no influence on the reduction of the interfacial tension between oil droplets at increasing droplet formation time (4.3.1.1). Emulsifiers reduce the interfacial tension between the oil droplets and the aqueous phase (2.1.2). The effectiveness of an emulsifier is reflected in the reduction of the interfacial tension and the adsorption time (speed) to the oil droplet surface (2.1.4). In this case, the emulsifying activity of the proteins and the protein-phospholipid complexes was not altered. The statement is confirmed by the unchanged oil droplet size distribution and the specific surface areas of the oil droplets in the emulsions (4.3.2.2). However, the emulsifying property of egg yolk is affected by the enzyme modification and high-pressure homogenization in the interfacial formation (discussion 4.3.3.2). The hydrolysed egg yolk generates a homogeneous thick and stable interfacial film. The high pressure homogenisation of hydrolysed egg yolk generates an even thicker and more stable interfacial film. Excessive pressure (such as 300 MPa) generates an even thicker but non-uniform interfacial film. This interfacial film reduces stability the oil droplets and hence also the emulsion (EFF in 4.3.2.5 and discussion in 4.3.3.4). Therefore, an excessive pressure homogenization of 300 MPa deteriorates the emulsifying activity of the hydrolysed egg yolk.
4.3.3.4 Influence of UHPH-Processing on the Stabilization Abilities of Egg Yolk in an Emulsion

The stability of the emulsion was determined in this work using the extractable fat fraction (EFF), the protein interfaces-occupancy and the stability tests. The EFF (4.3.2.5) and the protein interfaces-occupancy (4.3.2.4) describe the characteristics of the interface film of oil droplets. The CLSM images (4.3.2.6) are consulted to illustrate the results. The emulsion of conventional egg yolk had thin and less stable interfacial films, because the EFF was more than twice as high as in the other emulsions. Even though, the emulsion had a larger protein interfaces-occupancy like the hydrolysed emulsion. This difference is caused by the greater proportion of proteins at the interface (Figure 44), which only occupy a small part of the oil droplets interface due to its aggregated form (low solubility in the continuous phase) and therefore have only a small contribution to the stabilization of the interface (see 4.3.3.2). The emulsions prepared with modified egg yolk, untreated and 225 MPa homogenized, had stable interfacial films, as in agreement to the low fat extraction. These results were confirmed by the findings from the discussion 4.3.3.2. Moreover, the results of the protein interface-occupancy support the observation of the CLSM images (4.3.2.6), that the 225 MPa treated emulsion has an interfacial film thicker than the untreated emulsion. Furthermore, the results of EFF and protein interfaces-occupancy prepared with modified egg yolk treated at 300 MPa confirmed the observation of the CLSM (4.3.2.6). This emulsion had thick (i.e. largest protein interfaces-occupancy) but inhomogeneous interfacial films (i.e. largest fat extraction in the emulsions of hydrolysed egg yolk) in comparison to the other emulsions.

The stability test (4.3.2.3) describes the macroscopic stability of the emulsions with respect to the creaming and phase separation. All emulsions showed no phase separation of oil and water during the stability tests and were therefore stable.

The creaming (2.1.3) occurs due to the gravitational force and the difference in density between the continuous and disperse phase. The creaming is accelerated by oil droplet aggregation (flocs) and reduction in viscosity in the emulsion. The emulsion of conventional egg yolk had the lowest viscosity (4.3.2.1) and strongest droplet aggregation (4.3.3.2), accordingly a significant creaming was observed in this emulsion already on day 1 after production. The emulsions prepared with hydrolysed (PLA₂-modified) egg yolk showed on day 3 after preparation a low creaming due to the higher viscosity and the lower aggregation of droplets. The high-pressure homogenization of egg yolk stabilizes the emulsion against the creaming. The reason for the stabilization is the network formation of the protein-lysophospholipid complexes which increase the viscosity of the emulsion. The smallest creaming was observed in the emulsion prepared with the egg yolk treated at 300 MPa, although it had a less dense network (lower viscosity). This anomaly could arise due to the
fine distribution of air bubbles (4.3.2.3) in the emulsion. There is the possibility that the air bubbles stabilize by the highly folded protein threads (4.3.1.2) or the folded egg white (Heinzelmann et al. [1994] in 2.5 and discussion 4.1.2).

4.3.3.5 Influence of UHPH-Processing on the Rheological Characteristics of Egg Yolk in an Emulsion

Viscosity is an important quality characteristic of an emulsion. The emulsion prepared with hydrolysed (thermostable) egg yolk showed a higher consistency than the emulsion prepared with conventional egg yolk. The results from the Pilot-Tests 2 were then confirmed. The higher viscosity in the emulsion of hydrolysed egg yolk arises by the partial break-up of the granules (2.3.3), which enlarges the protein volume in the continuous phase and leads to a larger network formation. The high-pressure homogenization of hydrolysed egg yolks with 225 MPa further increased the consistency, which is explained by the SEM and CLSM images from chapter 4.3.1.2 and 4.3.2.6. This showed an increased protein network formation (cross-linking) after the high-pressure homogenization. The reduction of the consistency of the high pressure treatment at 300 MPa is based on the excessive denaturation of the protein formed networks. The reduction of the consistency of the emulsion was based on the excessive denaturation of the protein networks (4.3.3.1 and 4.3.3.2) formed by the high pressure treatment of egg yolk at 300 MPa.
5. Summary and Conclusions

Summary

The aim of this master thesis was to determine the effect that Ultra High Pressure Homogenisation (UHPH) processing has to the conventional homogenised and pasteurised egg yolk and the thermostable egg yolk (enzymatically modified by phospholipase A\(_2\) (PLA\(_2\))) with respect to the emulsification and stabilization ability in model-emulsions.

Based on the results of Marco-Moles et al. [2012], conventional egg yolk and also thermostable egg yolk (PLA\(_2\)-modified) were examined in the Pilot-Tests 1. The Pilot-Tests 1 characterized the Premixes (1:1 with physiological solution) of diluted conventional egg yolk and thermostable egg yolk regarding the changes in viscosity and stability behaviour in an O/W (50/50) model-emulsion with a protein content of 1.25% as affected by ultra-high pressure homogenization.

The Pilot-Tests 2 investigated the influence of different ratios (recipes) of oil to water (i.e. 10/90; 35/65 and 50/50) and different amounts of ultra-high pressure homogenized thermostable egg yolk (i.e. protein content: 1.25% and 1.625%) with respect to emulsifying, stabilizing and rheological behaviour in the emulsion. The emulsions were re-examined after 10 days, to determine whether the high-pressure homogenised yolk shows a different behaviour in stability than the untreated egg yolk.

The main focus of the investigations of the Main-Tests was to study the structural changes taking place in the egg yolk by ultra-high pressure homogenization and its effect on the microstructure of the emulsion (O/W; 50/50 with protein content of 2%) with respect to emulsifying, stabilizing and rheological behaviour.

The analysis of the Pilot-Tests 1 revealed an increase in the temperature of 15-20 °C / 100 MPa during the high-pressure homogenisation range studied (100, 150, 200, 250, 300 and 350 MPa) of the Premixes (conventional egg yolk and also thermostable egg yolk). The proteins of the conventional egg yolk underwent a severe denaturation at the homogenisation pressure of 350 MPa. The protein network of the thermostable egg yolk (PLA\(_2\)-modified) was protected from the entire unfolding of proteins by formation of the complex of Apo-LDL to the lysophospholipids and free fatty acids. The high-pressure homogenization of conventional egg yolk had a negative effect on the emulsion, because it reduced the consistency, regardless of the pressure level of homogenization. However, the homogenization treatment of the thermostable egg yolk at lower pressure (i.e. 100 to 250 MPa) unfolded proteins in the Premix to a degree, so that the stabilising behaviour increased significantly in comparison to the untreated. An exception is the high pressure...
treatment of thermostable egg yolks exceeding 250 MPa where the consistency decreased in comparison to the lower pressures perhaps due to the larger amount of unfolded proteins and excessive denaturation. The stability of the emulsions prepared with thermostable egg yolk was also significantly better than that of the emulsions prepared with conventional egg yolk.

The Pilot-tests 2 pointed out that the most important factor influencing the viscosity and stability of an emulsion was the concentration of oil in the recipe. The protein content in the emulsion and the previous high-pressure homogenization of egg yolk had a much lower influence. Furthermore, the high pressure homogenization of Premixes of egg yolk had barely an effect on the size distribution and specific surface area of oil droplets in emulsions. The repeated analysis of the emulsions after 10 days showed no major differences in the oil droplet size distribution and the specific surface area compared to the results after day 1. This means that the emulsion interfacial films of all analysed emulsions were stable and there was no coalescence and / or Ostwald-ripening on a larger scale.

The investigations of the rheological characteristics of egg yolk in an emulsion revealed that emulsion of hydrolysed egg yolk displayed higher consistency than the emulsion of conventional egg yolk. The higher viscosity in the emulsions prepared with thermostable egg yolk arises by the partial break-up of the granules, which enlarges the protein volume in the continuous phase and leads to a larger network formation of protein-lysophospholipid complexes. The high-pressure homogenization of thermostable egg yolk with 225 MPa further increased the consistency of the emulsion due to an increased protein network formation (cross linking). The high-pressure homogenization of thermostable egg yolk with 300 MPa showed a decreased consistency of emulsion in comparison to the already mentioned 225 MPa-emulsion. This reduction of the consistency might be associated to an excessive denaturation of proteins the hereby a lower dense protein network results.

The investigations of the stabilization abilities of egg yolk in an emulsion revealed that the emulsions prepared with conventional egg yolk had the largest creaming which is due to the thin and unstable interfacial film (droplet aggregation) and the low density of protein network (low viscosity). The reason for the smaller creaming was the network formation of the protein-lysophospholipid complexes which increase the viscosity of the emulsion. The thermostable egg yolk showed the smaller creaming in emulsion because of the already mentioned denser protein networks and the resulting higher viscosity. With increasing pressure the creaming of the emulsion was reduced.
Conclusion

The enzyme modification of egg yolk by phospholipase A₂ (PLA₂) increases the viscosity and stability of the model-emulsions compared to conventional egg yolk. Furthermore, phospholipase A₂ modification help protect the structure of egg yolk against the high forces and temperatures generated during the high-pressure homogenization processing. Moreover, the high-pressure homogenization processing induces protein modifications likely by unfolding the protein structure which results in an increased crosslinking of the proteins, lyso- and phospholipids and finally results in a denser network. This network and the improved stabilization of the interfacial films of oil droplets generates in the emulsion a higher viscosity and stability. However, the high-pressure homogenization of conventional egg yolk has a negative effect on the emulsion, because it decreased the viscosity, regardless of the pressure level of homogenization, and shows no improvement in the stability.

The homogenization pressure of 225 MPa was incorporated as the optimal processing conditions that allow the generation a good balance between viscosity and stability in emulsion. The pressure range of 100 to 150 MPa enabled the egg yolk to generate high viscosities and lower stability in emulsion and vice-versa to the pressure range of 300 to 350 MPa.

6. Future Recommendation

The ultra-high pressure homogenization processing of the thermostable (PLA₂-modified) egg yolk is an alternative way to increases the viscosity and stability of an emulsion. The ultra-high pressure homogenization processing of entire emulsion systems such as mayonnaise or salad cream with enzyme modified egg yolk would increase the viscosity and stability considerably due to the greatly reduced oil droplet sizes (down to 0.2 μm; Donsi et. al. [2009], and 2.4.1) and the enlargement of the specific surface area (Schuchmann [2005] in Viscosity 2.1.3). Further, the thermostable egg yolk processed by UHPH improves the stabilization of the interfacial films of oil droplets and the continuous phase between the oil droplets with a dense protein network. This could enable, for example, the fat reduction in mayonnaise without the addition of (extra) stabilizers. Furthermore, the emulsions could be microbiologically stable without the need of subsequent pasteurization. The high temperatures (Figure 15) occurring during the UHPH-processing could serve as a pasteurization process (salmonella typhi, D₇₅ 0.006: 250 MPa, 75 °C). The processing of the
product to get a long shelf life should include a customized heat retention time (temperature-soaking zone) and an aseptic packaging (with subsequent cooling).

The previous works [Floury et al.; 2000 and Marco-Molés et al.; 2012] increased the viscosity and stability of the emulsions without the use of a thermostable emulsifier / stabilizer, just based on the reduction of the oil droplets. The proposed processing based on combination of high-pressure homogenization of entire emulsion systems together with the use of thermostable egg yolk could result in an improved product and thus has a potential for market opportunity.
7. References


## 8. Appendix

Table 4. Flow Behaviour Index by Ostwald de-Waele of all tested O/W model emulsion, produced with the Premixes of PLA2-modified egg yolk which was treated by HPH at different pressures, after 1 and 10 days at 7 °C.

<table>
<thead>
<tr>
<th>Emulsions</th>
<th>Homogenisation Pressure [MPa]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>O/W-10/90-1,25_day1</td>
<td>1,03</td>
</tr>
<tr>
<td>O/W-10/90-1,25_day10</td>
<td>0,94</td>
</tr>
<tr>
<td>O/W-10/90-1,625_day1</td>
<td>0,85</td>
</tr>
<tr>
<td>O/W-10/90-1,625_day10</td>
<td>0,94</td>
</tr>
<tr>
<td>O/W-35/65-1,25_day1</td>
<td>0,63</td>
</tr>
<tr>
<td>O/W-35/65-1,25_day10</td>
<td>0,75</td>
</tr>
<tr>
<td>O/W-35/65-1,625_day1</td>
<td>0,63</td>
</tr>
<tr>
<td>O/W-35/65-1,625_day10</td>
<td>0,77</td>
</tr>
<tr>
<td>O/W-50/50-1,25_day1</td>
<td>0,57</td>
</tr>
<tr>
<td>O/W-50/50-1,25_day10</td>
<td>0,62</td>
</tr>
<tr>
<td>O/W-50/50-1,625_day1</td>
<td>0,53</td>
</tr>
<tr>
<td>O/W-50/50-1,625_day10</td>
<td>0,62</td>
</tr>
</tbody>
</table>
Table 5. Effect of oil concentration (10, 35 and 50%) and HPH (200, 225, 250, 300 MPa) treated protein content (1.25 and 1.625%) in O/W emulsions on their sauter mean diameter, dispersion index (SPAN) and specific surface area, after 1 and 10 days at 7 °C.

<table>
<thead>
<tr>
<th>Sauter mean diameter [μm] of Emulsion</th>
<th>Homogenisation Pressure [MPa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-90_1.25_day1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2,730</td>
</tr>
<tr>
<td>10-90_1.25_day10</td>
<td>2,755</td>
</tr>
<tr>
<td>10-90_1.625_day1</td>
<td>2,694</td>
</tr>
<tr>
<td>10-90_1.625_day10</td>
<td>2,774</td>
</tr>
<tr>
<td>35-65_1.25_day1</td>
<td>2,948</td>
</tr>
<tr>
<td>35-65_1.25_day10</td>
<td>2,634</td>
</tr>
<tr>
<td>35-65_1.625_day1</td>
<td>2,552</td>
</tr>
<tr>
<td>35-65_1.625_day10</td>
<td>2,752</td>
</tr>
<tr>
<td>50-50_1.25_day1</td>
<td>2,852</td>
</tr>
<tr>
<td>50-50_1.25_day10</td>
<td>2,757</td>
</tr>
<tr>
<td>50-50_1.625_day1</td>
<td>2,343</td>
</tr>
<tr>
<td>50-50_1.625_day10</td>
<td>2,455</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SPAN [-] of Emulsion</th>
<th>Homogenisation Pressure [MPa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-90_1.25_day1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2,057</td>
</tr>
<tr>
<td>10-90_1.25_day10</td>
<td>2,065</td>
</tr>
<tr>
<td>10-90_1.625_day1</td>
<td>2,473</td>
</tr>
<tr>
<td>10-90_1.625_day10</td>
<td>2,231</td>
</tr>
<tr>
<td>35-65_1.25_day1</td>
<td>1,943</td>
</tr>
<tr>
<td>35-65_1.25_day10</td>
<td>2,007</td>
</tr>
<tr>
<td>35-65_1.625_day1</td>
<td>2,054</td>
</tr>
<tr>
<td>35-65_1.625_day10</td>
<td>1,968</td>
</tr>
<tr>
<td>50-50_1.25_day1</td>
<td>1,610</td>
</tr>
<tr>
<td>50-50_1.25_day10</td>
<td>1,709</td>
</tr>
<tr>
<td>50-50_1.625_day1</td>
<td>1,809</td>
</tr>
<tr>
<td>50-50_1.625_day10</td>
<td>1,741</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Specific Surface Area [m²/g] of Emulsion</th>
<th>Homogenisation Pressure [MPa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-90_1.25_day1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2,39</td>
</tr>
<tr>
<td>10-90_1.25_day10</td>
<td>2,37</td>
</tr>
<tr>
<td>10-90_1.625_day1</td>
<td>2,46</td>
</tr>
<tr>
<td>10-90_1.625_day10</td>
<td>2,35</td>
</tr>
<tr>
<td>35-65_1.25_day1</td>
<td>2,21</td>
</tr>
<tr>
<td>35-65_1.25_day10</td>
<td>2,48</td>
</tr>
<tr>
<td>35-65_1.625_day1</td>
<td>2,56</td>
</tr>
<tr>
<td>35-65_1.625_day10</td>
<td>2,37</td>
</tr>
<tr>
<td>50-50_1.25_day1</td>
<td>2,29</td>
</tr>
<tr>
<td>50-50_1.25_day10</td>
<td>2,37</td>
</tr>
<tr>
<td>50-50_1.625_day1</td>
<td>2,78</td>
</tr>
<tr>
<td>50-50_1.625_day10</td>
<td>2,66</td>
</tr>
</tbody>
</table>
9. Selbstständigkeitserklärung

Erklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Hannover, Januar 2013

______________________________

Alexander Meißner