Extraction of protein mixture from rapeseed for food applications

MSc Thesis
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ABSTRACT

Increasing demand on protein due to increasing world population, prompts to search alternative sources and environmental friendly processes. Rapeseed is a promising protein source composed of mostly oil and protein. The current conventional method contains a de-fatting step using hexane as a solvent. The objective of this thesis is to design a green process (aqueous extraction process) to extract rapeseed protein mixtures without de-fatting and to investigate the production of emulsions using this protein mixture. In the aqueous extraction process, only water was used as a solvent. To extract the protein mixture, alkaline extraction and isoelectric precipitation were applied. As a result, a protein mixture with a protein purity of 60 wt% was obtained. The protein characterization was done by SDS-PAGE, where all 3 major rapeseed protein fractions were identified. The obtained protein mixture was used as emulsifier in a solution with 0.5 g protein/100 mL. pH of the emulsions were adjusted to 7 and 3.8, to compare the results with dairy and dressing type applications. In food industry stability of emulsions are of primary importance. Physical stability of emulsions are determined by droplet size distribution, droplet charge, stability against creaming and rheological characterization. The stability was observed during 5 days of storage and the emulsion formed at pH 7 was found to be more stable than at pH 3.8. Protein mixtures obtained by aqueous extraction process may have a high potential in food applications.

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1. Introduction

Increasing world population accompanies scarcity among usable nutritional sources in addition to environmental problems. Proteins are one of the building blocks for the human diet, animal feed, and also industrial applications. In parallel with an increasing world population and increase in wealth, the demand for protein rises, especially animal based protein. However, consumption of animal based protein has negative impacts, such as greenhouse gas emissions, excess water usage, air and water pollution, and habitat loss[11]. These conditions lead consumers to shift their diet more towards a plant protein rich diet, and to search for novel protein sources and sustainable processing strategies to extract them[14].

One of the promising protein sources are agricultural crops, such as oilseeds. Oilseed crops are cultivated for their oil and protein. The extracted oil is used for non-edible purposes such as biodiesel or edible purposes, such as food dressings or cooking oil. The remaining solid layer after oil extraction is called de-fatted meal (cake), which is rich in protein and mostly used as feed than for food applications. The three most promising oilseed crops worldwide are soy, rapeseed and sunflower due to their high nutritional value[9,10]. According to the United States Department of Agriculture (USDA), rapeseed is third in worldwide oil seed production[1] and according to the Food and Agriculture Organization of the United Nations (FAO), it is the most important source for vegetable oils in the world after soybean[2]. Furthermore, in the last decade rapeseed production increased along with the rapeseed oil demand[17].

The reason of focusing on rapeseed rather than other oilseed crops is that it can grow under wider climate conditions in Northern and Western Europe, has gelling characteristics at lower temperatures, a high fatty acid content, a consistent thermal and storage stability and a well-balanced amino acid composition[12,23,34]. In comparison to the market leader soybean, rapeseed has a lower carbon footprint (521 g $C0_2$-eq/kg rapeseed meal vs. 578 g $C0_2$-eq/kg soybean meal)[34].

Rapeseed is mainly composed of oil (37-50%) and proteins (15-26%)[12,13,24,30]. Current protein extraction processes are focused on producing high purity products, namely rapeseed protein isolates (RPI) and rapeseed protein concentrates (RPC) from de-fatted rapeseed meal (cake) by aqueous-alcoholic protein isolation recovery procedures. Where, the driving force is the solubility differences of major rapeseed proteins due to pH or ionic strength[12,24,26,29,30,32]. Hexane usage in the de-fatting step to extract oil and intensive pH changes bring along poor functional products and limitations for feed and food applications because of health effects and environmental safety. To overcome those malfunctions and to gain high purity products, non-sustainable and high cost methods are being applied. According to studies for food applications on oil-water surface rheology and emulsion stability, mixtures have same or even better functional properties than the pure products[43,44]. Therefore, a mild process that extract a mixture of main components has been proposed as an alternative to current extraction processes for food applications, namely Aqueous Extraction Process (AEP) [43,43,50,51].

In AEP, de-fatting and purification steps are skipped and only water is used as a solvent to avoid harsh conditions, hazardous chemicals and product denaturation. As a result of the extraction process, a high amount of protein, a significant amount of oil and a minor amount of rest of the biomass can be extracted as mixture. In AEP, oil is gathered as oil bodies (OB's) that are released into aqueous media right after the protein diffusion[44,50,51]. On the contrary, in current conventional processes, protein remain in the de-fatted meal within a mixture of carbohydrates and fiber while oil dissolves into the solvent. In this thesis, the obtained protein mixture by the AEP was used to make an oil-in-water emulsion to understand the functional properties for further food applications. The waste streams were not a concern of this thesis. However, they can be considered as a feed source due to their nutritional value.

Emulsion based food products are one of the food applications where the protein from oilseed crops can be used. In general, for all food products, stability is a key factor. Chemical and physical stability depends on the oilseed composition and the extraction method[45]. Therefore, interfacial properties and structure characteristics of specific fractions should be studied to understand the applicability. In case of emulsion food products, the component must be incorporated into the emulsion food products. Since the de-fatting step using hexane was omitted, the likelihood of denaturation of substances are decreased and protein fractions can be gathered in their native form[43,44]. Due to simple, mild and green extraction, omitted purification treatment steps and water usage as solvent, it is expected that a high physical and chemical stability and nutritional value can be achieved.

2. Scientific Objective

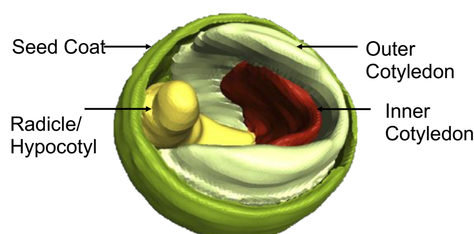
1. To design a process for extraction of protein mixtures from non-defatted rapeseeds.
2. To investigate the production of emulsion stabilized by the aqueously extracted protein mixtures.

3. Background

3.1. Composition of Rapeseed

Rapeseed is a member of family Brassica. There are three major species namely *B. napus* (cultivated in Europe and Canada, having a black or reddish color), *B. rapa* (cultivated in Canada, having a black or yellow color), *B. juncea* (cultivated in Canada, having a yellow color). The difference is mainly in oil content[33]. Depending on the planting season, either fall or spring, rapeseed is called winter rapeseed or spring rapeseed, respectively[22,27]. It can grow with less sunlight and at lower temperatures compared to other crops[22,27]. There are two types of rapeseed: industrial and edible (which is also called Canola). The development of the edible rapeseed, Canola, began in Canada and Europe, with the aim to improve the quality of extracted oil by having less than 2% of erucic acid and having a low level of glucosinate in the de-fatted rapeseed meal (as 30 micromoles/g meal)[19,24]. Erucic acid and glucosinate make rapeseed non-edible. For instance, the non-edible industrial rapeseed has 55% of erucic acid and high levels of glycosinolate[27].

The three major parts of the rapeseed are: embryo (composed of cotyledon, hypocotyl and radicle), endosperm and seed coat (surrounds the embryo and the endosperm)[29]. (Figure 1).



The highest protein (approximately 25 %) and oil (approximately 55%) content are in the cotyledon compared to whole seed and seed coat[29]. The weight of the seed coat is inversely proportional to the seed quality. The seed coat contains N-based components which are proteins and non-proteins, where the major contributors of non-proteins are glucosinolates and tri-amine compounds[29].

Figure 1. A cut-away three-dimensional model of rapeseed[35]

Rapeseed as a whole is mainly composed of oil and proteins, followed by carbohydrates, lignin, ash and secondary metabolites (phenolic compounds). Studies show different ranges for the composition (Table 1). The content depends on the cultivar, temperature and weather changes during maturation period and amount of sulphur within the seed[12].

Table 3.1. Average of Reported Composition of the Edible Rapeseed (Canola)*[12,13,18,24,29]

Oil (%)	Protein (%)	Carbohydrates (%)	Lignin (%)	Ash (%)
43	21	14	8	5

**Component compositions given in dry matter content (dm%). Average content calculated using the average of available data, which will be used as a comparison for the results of the composition analysis.*

The oil content in the rapeseed consists mostly of fatty acids[28]. The protein content consists of three major protein fractions: the two storage protein groups (SSP's): cruciferin (as globulin) and napin (as albumin), which are major proteins of the cotyledon extract, and a membrane protein (structural protein), namely oleosin[8,12]. The focus of this research is on the protein content and protein fractions, a detailed explanation given in Part 3.2. The carbohydrates composition of rapeseed can be divided into 3 groups: soluble sugars, insoluble carbohydrates and fiber[33]. Sucrose is the predominant sugar; cellulose, hemicellulose and pectin[30] form the insoluble carbohydrate fraction[33]. Fibers are found in the seed coat, endosperm, and cell walls of the cotyledon cells. They contribute mostly to the carbohydrate fraction[30]. Phytates are another component that associate as insoluble crystals in the

protein storage vacuoles (PSV) in the seed. They are related to carbohydrates in terms of structure[33]. Salts of Ca, Mg and K or phytic acid are referred to phytates[30].

3.2. Rapeseed Protein

3.2.1. *Amino Acid Content*

Rapeseed, a plant based protein source, is a promising alternative to animal based protein sources. In plant based proteins, proportion and variety of the amino acids depend on type of the plant. Generally, plant proteins lack of one or more amino acids, but plant based protein diet is more sustainable and have health advantages[37].

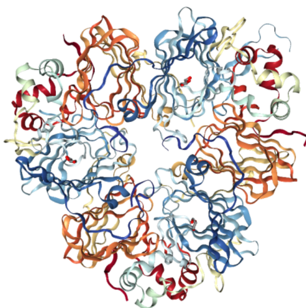
Rapeseed known as having a better balanced amino acid composition compared to other oilseeds contain eight out of these nine essential amino acids except tryptophan[19]. Compared to soy, rapeseed protein has a higher cysteine and methionine content, which are sulphur containing amino acids[26]. It should be considered that there are limits for amino acid concentrations in human diet. Surplus or lack of amino acids can cause health issues. The protein extraction method influences the concentration of amino acid compositions[19,24]. Therefore, protein extraction from rapeseed should be designed carefully depending on the application of the product in food sector.

3.2.2. *Protein Fraction*

Proteins in plant seeds can be located in the cell as protein bodies, in the membrane, or can be structural. To obtain proteins that are in the cell, the cell wall should be ruptured by a pre-treatment. To obtain membrane proteins, lipid layers should be separated[38]. Rapeseed has three main protein fractions, namely crucifein, napin and oleosin. The primary, secondary and tertiary structures of them differ highly, thus they have different functionalities and properties[29]. The predominant storage proteins, cruciferin and napin, are located in protein storage vacuoles of embryonic tissues of the seed[29]. In Europe, the ratio of cruciferin to napin varies between 0.6 to 0.2 depending on erucic acid and glucosinolate levels[30]. Oleosin is a structural protein that is found in the membrane of the organelle called oil body (OB) which is the oil storage of the seed[24].

Cruciferin

Cruciferin is a 12S globulin and it belongs to the cupin superfamily[12,29]. It is the predominant protein in rapeseed, responsible for 60 % of total protein content[12,29]. Cruciferin has a molecular mass of 300-360 kDa and an isoelectric point of pH 7.25 [29]. It is a neutral protein, which can act as gelling agent in its native form[12,29]. It is a salt soluble protein[29]. In despite of the limited information about their bioactivity, it is stated that amino acids of cruciferin play an important role in gastro-intestinal digestion[30].

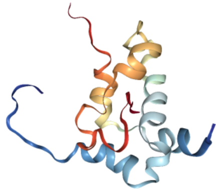


The cruciferin has six subunits structured in two trimer units (Figure 2). The two trimer units combine with each other by non-covalent bonds as hydrophobic, electrostatic, hydrogen, Van der Waals or hydrogen bonded salt bridges. Each protomer composed of two polypeptide chain linked with a disulfide bond, where the polypeptides are acidic α-(approximately 40 kDa, 254 to 296 amino acids) and basic β-(approximately 20 kDa, 189 to 191) amino acids [24].

Figure 2. Crystal structure of cruciferin[39] (rcsb.org)

Napin

Napin belongs to the prolamin superfamily[29]. It is a 2S albumin (or 1.7S), which is the second major contributor to the protein content, comprising 20% of total protein in the rapeseed[12]. The amount of albumin is related to the amount of sulphur compounds presented in the seed, which are related to sulphur containing amino acids such as cysteine and methionine[12]. Napin is known as the nitrogen and sulphur supplier of the seed, due to its ability to mobilize during germination; having high content of protein bodies and substantial amino acid composition[30]. Furthermore, they show antifungal activities in rapeseed[30].



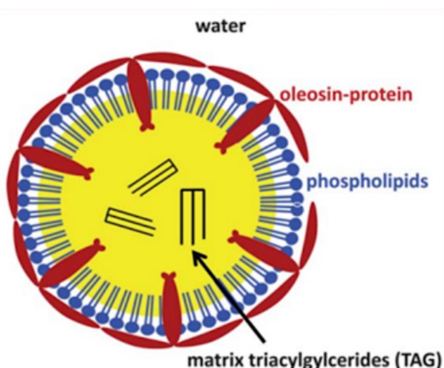
Napin has a molecular mass of 8-15 kDa[12]. It has a strong alkalinity and can be solubilized in water[12]. Studies show that napin has high thermal stability and high structural stability among wide pH ranges, which can be soluble at low pH's such as pH 2-4[29,30]. At pH 11, napin does not have a net electric charge[29].

Figure 3. Crystal structure of napin[40] (rcsb.org)

The mature napin structure composed of one small (4-6 kDa) and one large (10-12 kDa) polypeptide chain linked via four disulfide bonds (two inter- and two intra- chain disulfide bonds) (Figure 3). The large polypeptide chain is composed of two intra-chain disulfide bonds between cysteine residues, which stabilize the napin molecule with four disulfide bridges[24].

Oleosin

Oleosin is a structural (membrane) protein that compromises 2-8% of the total protein in rapeseed. It is an alkaline protein with a low molecular weight around 15-20 kDa.[12,24,42]. Oleosin is also known as the dominant oil body protein (OBP) in rapeseed which associates with oil bodies (OB) [12,24]. OBP's are subcellular organelles that stabilize OB's[38]. They have a long hydrophobic and a hydrophilic domain, which can associate with the lipid phase and the surface of the OB, respectively[24].



OB's are subcellular hydrophobic organelles that store oil, which consist of storage triacylglycerols (TAG) and are surrounded by phospholipids (PL) and oleosins (Figure 4)[38]. TAG, PL and oleosin content varies due to the size of OB's, where it is in the rage of 0.6-2.0 μm. The size of OB's varies depending on the type of the oilseed, environmental factors and availability of nutritional requirements such as nitrogen for oleosins and phosphate for PL[38].

Figure 4. Proposed structure of oil bodies[41]

The isoelectric point of OB's is determined in the range of pH 5.7 to 6.6, which means that at neutral pH, the surface charge is negative. The isoelectric point varies among the seed types[38].

TAG acts as a carbon and energy source for the seed germination and growth[38]. The outer surface of TAG, phospholipid-protein complex, provides physical and chemical protection against environmental stresses, like moisture and temperature changes and presence of oxidative reagents. Oleosin act as a size-regulator for oil bodies, where OB size is inversely proportional to oleosin content. Furthermore, it is responsible for freezing tolerance, which creates a benefit for the seed that it can grow at low temperatures and survive harsh winter conditions [42]. Oleosin has a negatively charged surface. This is

why it can stabilize and prevent coalescence of the OB's by creating steric hindrance and electrical repulsion[24,42]. It has an N-terminal (N) amphipathic domain (having both hydrophobic and hydrophilic characterization), a central hydrophobic domain and a C-terminal (C) amphipathic α-helical domain. N and C domains are expected to ease the association with phospholipid surfaces[38].

3.3. Rapeseed Market

Worldwide, the three most promising oilseed crops are: soy, rapeseed and sunflower due to their high nutritional value[9,10]. Global rapeseed production in 2017/2018 is estimated as approximately 70 million metric tons (MMT) by the United States Department of Agriculture (USDA)[1] . According to Food and Agriculture Organization of the United Nations (FAO), rapeseed is the second leading source for vegetable oils in the world, after soybean[2] (Figure 5). According to FAOSTAT, from 1994 to 2016, China was the top rapeseed producer (11.8 million MT), followed by Canada (10.4 million MT) and India (6.3 million MT) as an average through years[16]. In the European Union (EU), where rapeseed is used mainly as a source for biodiesel, Germany and France are the main producers with approximately 4 million MT[16]. Therefore, from an economical point of view, rapeseed is a promising alternative as protein source compared to other vegetable oilseeds and animal products.

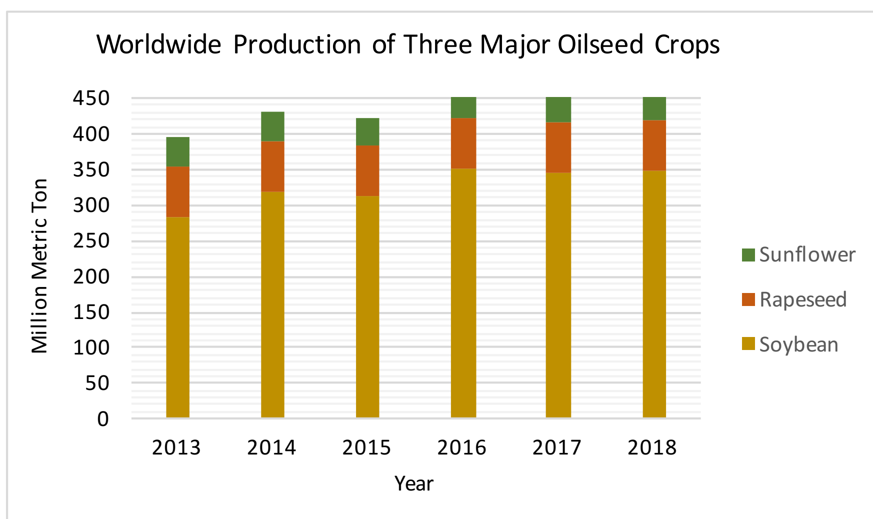


Figure 5. Worldwide production of three major oilseed crops, data gathered from Office of Global Analysis (OGA)[15]

3.4. Current Application and Studies

Currently industrial production with rapeseed is mainly focused on oil extraction for edible and non-edible usage. The extract, which is called de-fatted meal (cake), is containing a high percentage of valuable protein which is utilized as a high protein biomass source in aquaculture and livestock industries[9,18,19]. High production costs prevent industries to take advantage of the protein in rapeseed for food applications rather than feed. Therefore, studies must be continued to reduce production costs and increase yields.

In industry, the rapeseed oil is utilized for biodiesel since it is a renewable energy source, environment friendly with low cost extraction route. Having high yields of oil per hectare (1,188 to 1,497 litres per hectare), gelling characteristic at lower temperatures, and high fatty acid content compared to the other vegetable oils make the rapeseed oil a competitive feedstock for biodiesel production. For

example, in Europe, non-edible rapeseed is the most common used oilseed for biodiesel production[12,23]. Furthermore, the low saturated fat content, nutritional value and consistent thermal and storage stability of rapeseed oil enables in addition to cooking oil a wide application range as a food additive such as from mayonnaise and salad dressings to margarines and bakery products[20].

Research for food application by using RPI and RPC demonstrated that the rapeseed protein can potentially be used in bakery products, beverages, dairy and egg substitutes, processed meat products, salad dressings and sauces flavourings[12,19,24]. The reason is that RPI and RPC show high nitrogen solubility at neutral pH[26]. As an example, RPC is a promising source for replacing milk and other vegetable proteins in sausage[12,19,24]. Moreover, according to WHO, even the daily protein intake can be achieved by RPI and RPC since de-fatted rapeseed meal contains recommended amount of essential amino acids[3]. However, de-fatted rapeseed meal also contains glucosinolates, phenolics, phytates and high amount of fibers, which have negative effects on digestibility, color and taste of the product[19]. Therefore, extensive studies are ongoing for food applications.

Furthermore, studies with RPI for films with water barrier properties, hydrogel as superabsorbent, protein based surfactants/foams/interface active molecules, protein-based plastics, protein-based adhesives and nanoparticles for control delivery of bio actives gave positive results[19,24].

3.5. Food Emulsions

Emulsions consist of two immiscible liquids, namely dispersed phase and continuous phase (Figure 6.a). Where one of the phases is dispersed as droplets into the other one by shearing force (Figure 6.b)[59]. The most common emulsion type is oil-in-water (o/w), such as mayonnaise and food dressings. O/w emulsions comprise of the dispersion of small oil droplets into the aqueous phase by homogenization. Homogenization is referred to as an intense mechanical agitation in food industry (see Figure 6.c for emulsified state after homogenization) [45]. Food emulsions have a large phase boundary or interfacial area between continuous and dispersed phases, which contains free energy. The system tries to reduce the interfacial free energy by reducing the interface. Therefore, to increase the interfacial area an external force is needed[59].

Emulsions are unstable systems; their physicochemical properties can change over time due to further treatment and/or storage, since oil droplets tend to aggregate due to their relatively weak electrostatic repulsion forces with respect to each other. This can lead to phenomena such as partial separation, flocculation and coalescence which are undesirable for food applications (Figure 6.d). Stabilizers are used to prepare kinetically stable emulsions. Emulsifier is a type of stabilizer, which prevents aggregation of oil droplets due to its surface-active characteristics. Surface-active substances are creating a protective coat around the droplet by adsorbing the surface of it[44,45]. Emulsifiers lower the surface free energy by adsorbing phase surface, which lowers surface tension and diminishes the interfacial tension between the two interfaces of dispersed and continuous phases. Besides, reduction of interfacial tension and interface deformation become easier[59].

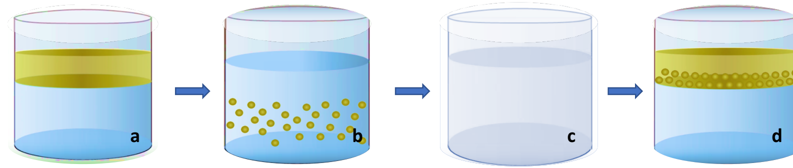


Figure 6. States of an emulsion. a: dispersed phase (oil) and continuous phase (water) before application of shearing power. b: Oil droplets are dispersing into the water phase. c: Emulsified state after homogenization. d: Thermodynamically unstable emulsion after storage period.

Proteins are one of the most preferred emulsifiers, since they can enhance physical and chemical stability of oil droplets within the aqueous phase[44,45]. In emulsions, proteins have the capability to enhance the strength of repulsive steric forces between the droplets that are near to each other. This enhancement cause high stability against coalescence, which is needed for long storage periods[45]. Furthermore, proteins are advantageous emulsifiers since they can rapidly absorb, change and re-arrange the newly created o/w interface. Due to their intermolecular interactions, proteins can form a cohesive and viscoelastic film over the surface of the oil droplet in an emulsion[19]. To form a protein emulsion, adsorption at the interface (usually occurring via hydrophobic interaction), reduction of interfacial energy and surface denaturation are required. Protein denaturation at the interface provides a stable conformation and reduces interfacial free energy[30]. Therefore, AEP will provide a stability advantage since the product will consist of a mixture of protein fractions which are cruciferin, napin and oleosin. Furthermore, recent researches[67] stated that in emulsions protein mixtures provide higher stability over oil droplets rather than pure protein isolates.

4. Materials and Methods

4.1. Materials

Food grade rapeseed was purchased from Ragt (Ragt Semences, France) and canola oil was gathered (Albert Heijn, The Netherlands) Sodium hydroxide (NaOH) was purchased from Sigma Aldrich (Sigma, USA). Hydrochloric acid (HCL, 37 % v/v) was purchased from AnalaR NORMAPUR (France). Petroleum ether 40-60 ^{o}C PEC grade was bought from ACTU-ALL (ACTU-ALL Chemicals BV, The Netherlands). All chemicals were analytical grade. In all experiments, demineralized water was used except buffers, which was MiliQ water obtained from Merk Milipore (Merk, Germany). All the experiments were carried out at room temperature (23 °C) and all the analyses were performed in triplicate.

4.2. Aqueous Extraction

Studies regarding AEP is mostly based on soybean for simultaneous oil and protein recovery [50,51]. For rapeseed, the focus is mostly on the oil extraction or pure protein isolation and/or concentration from de-fatted rapeseed meal. There is no other study for the protein extraction from whole rapeseed using AEP method. Therefore, in this study, the extraction route was based on the oil extraction by AEP [49,50,51]. Several modifications were done in order to gain a product that consists of mainly proteins. The modifications were influenced by previous studies that focus on protein isolation method[12,19,29,32,48]. The de-hulling and de-fatting steps were skipped to investigate the applicability of food emulsions in a simple, sustainable and mild way (Figure 3.1). This study does not concern the optimization of AEP process.

4.2.1. Protocol

Whole seeds were pulverized using an universal mill, IKA M20 (IKA-Works, Germany), for 70 seconds at 20,000 rpm. Rapeseed flour was soaked in demineralized water with a ratio of 1:7 (w/v), at room temperature. pH was adjusted to 8 using 1.0 M NaOH. pH was adjusted every 15 minutes to solubilize proteins in rapeseed as much as possible. Meanwhile the mixture was continuously stirred for 2 hours by an overhead stirrer (IKA EUROSTAR 40 digital, Germany), at 150 rpm. The mixture was stored in refrigerator (4 ^{o}C), overnight. The next day, the mixture was subjected to intensive agitation with a blender (Philips, The Netherlands) for 1 minute at maximum speed. After blending, the pH was adjusted again to 8. The blended mixture was filtered through two times folded cheesecloth (Local market, the Netherlands) to collect solubilized protein. The remaining solid phase was mixed with demineralized water with a ratio of 1:7 (w/v) and the mixture was blended and filtrated again. The two permeates were mixed with an overhead stirrer at a speed of 150 rpm for 10 minutes to get a homogeneous mixture and the pH was again adjusted to 8. The mixture was subjected to two centrifugations (Sorvall™ Legend™ XFR, Thermo Scientific, USA). The first centrifugation was run at 3,000 xg for 15 minutes at 4°C. The centrifugation process creates three streams: an oil fraction containing stabilized-oleosomes (supernatant/cream), an aqueous phase (subnatant), and a small residual solid phase (pellet) that primarily consists of cellulosic and hemicellulosic materials from the hulls. The pellet was removed and the cream and subnatant were collected and mixed with an overhead stirrer. The mixture was then subjected to a second centrifugation, run at 10,000 xg for 30 minutes at 4 ^{o}C. The cream and pellet were removed and the subnatant was recovered and stored at 4 ^{o}C, until further use.

To obtain target protein fractions, acidic precipitation was applied according to the isoelectric point (pI) of the recovered liquid phase. To indicate the isoelectric point, a ζ-potential measurement was executed and the pI was determined from the titration curve.

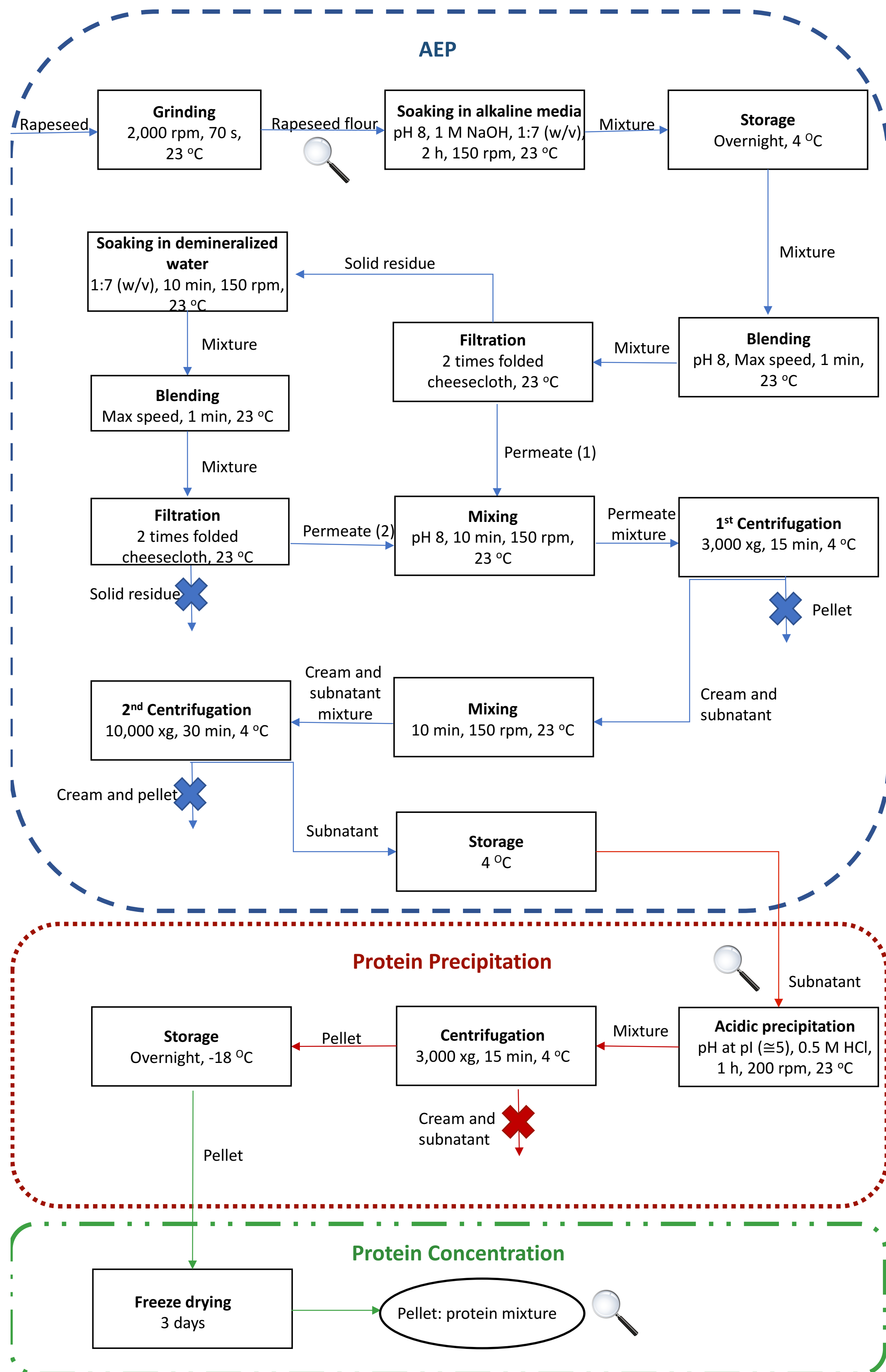


Figure 3.1. Flowchart of protein extraction process, the cross symbolizes the waste stream and the magnifier symbolizes further analyses

ζ-Potential measurement and titration curve

An aliquot (10 mL) of subnatant was first filtrated using filter paper circles, 604 grade, 25 μm pore size (150 mm) (Whatman Schleicher Schull GmbH, Germany) and a funnel, to get a clear solution. Then the subnatant was diluted with MiliQ water with a ratio of 1:100 (v/v).

The ζ-potential was measured in order to determine the magnitude of the electrostatic or charge dependent repulsion or attraction between particles. Measurement was done using a dynamic light scattering instrument (Malvern Zeta Sizer, Nano ZS, Malvern Instruments Ltd, UK). The reflective index was set for protein, at 1.450.

A titration curve was plotted to determine the isoelectric point (pI) of the subnatant. For pH adjustment, an auto titration was used (Malvern Instruments Ltd, UK). The pH was adjusted using NaOH (0.1 M and 0.5 M) and HCl (0.5 M) solutions. The pH range was between 2 to 12 with increments of 0.5. Each measurement was done in 12 runs with 3 replications. The determined pI was used to precipitate the target protein fractions.

Protein precipitation

The liquid phase was collected from the refrigerator and the isoelectric precipitation was performed using 0.5 M HCl. The pH adjustment was done according to the obtained data from the titration curve. Adjustment with HCl continued until stabilization, meanwhile the solution was continuously stirred with an overhead stirrer (IKA EUROSTAR 40 digital, Germany) at 200 rpm. After pH stabilization, the samples were centrifuged (Sorvall™ Legend™ XFR, Thermo Scientific, USA) at 3,000 xg for 15 minutes, at 4 °C. The pellet was collected and spread through in a plastic box to get a maximum surface area for the subsequent freeze-drying step. Then the pellet was stored in a freezer (-18 °C), overnight.

Freeze Drying

In order to preserve the stability of proteins, lyophilisation was applied by using a freeze dryer (Christ, Germany). The pellet was collected from the freezer after a storage period and placed into the freeze dryer. The plastic box was covered with laboratory tissue that was two times folded to avoid moisture during the process. Freeze drying took three days: 48 hours of main drying (0.011 mbar) and 24 hours of final drying (0.0010 mbar). After three days, the freeze-dried pellet was collected and stored in a Scott bottle sealed with para-film and a lid, after which it was stored at 4°C until further analysis and production of emulsions.

For the composition analysis, standard methods of AOAC were used to indicate moisture, oil and protein contents which were performed in triplicate.

4.3. Composition Analysis

Moisture content

The moisture content of rapeseed was determined in rapeseed flour. One gram (1 g) of ground rapeseed was dried in an incubator (Memmert Universal Oven, The Netherlands) at 50 °C, overnight. Dried samples were placed into a desiccator (Duran DN 300, Germany) to avoid moisture generation during cooling down. The moisture content in percentage was determined gravimetrically, where the difference of sample weight before and after the drying was divided by the sample weight. Analysis was performed in triplicate. (AOAC 24.003).

Oil content

The oil content of rapeseed was determined in rapeseed flour and in protein mixture using a Soxhlet apparatus, Behrotest EZ 100 H (Behr Labor Technik, Germany). The extraction run for 12 hours. The modified standard method of AOAC[57] was applied and the analysis was performed at room temperature, in triplicate. (AOAC 7.060-7.062).

Samples were dried before the oil extraction to avoid moisture, since some water-soluble materials would be extracted and give false measurement in terms of oil content. For rapeseed flour, oven drying was used, as explained in the moisture content part; for protein mixture, the freeze-dried product was used. One gram (1 g) of each sample was placed into cellulose extraction thimbles, 30 mmx80 mm (GE Healthcare Life Sciences Whatman™, UK). Oil was extracted from the samples by petroleum ether (200 ml/sample). To control boiling and to avoid superheating, 5 boiling chips (Lamers&Pleuger BV, The Netherlands) were added into each round bottom flask that contained the solvent. Heaters were set at 70 % during the process. After the oil extraction, oil containing round bottom flasks were transferred into an incubator (Memmert Universal Oven, The Netherlands) set at 50 °C for 1 hour to evaporate the excess solvent. The oil content (%) of the samples were determined gravimetrically, where the difference of round bottom flask weight before and after the oil extraction was divided by the sample weight.

Protein content

The Kjeldahl method was used to determine the protein content of rapeseed flour and gathered protein mixture after freeze drying by using block digestion and fume suction systems (Gehardt, Germany). The titration was done by a compact titrator for Stat applications (Metrohm, Switzerland). In general, the Kjeldahl method is used to measure nitrogen content in a sample. To convert nitrogen to crude protein, a conversion factor of both 5.7[12,33, 46, 47] and 6.25 were used. The factor 6.25 was used to compare the data with similar studies and with similar oilseed crops.

Required sample amount was calculated according to the estimated protein content (1 mg protein/sample). Samples were dried as stated in the moisture content part, to avoid faulty measurement caused by moisture. The analysis was done according to the FBR department protocol in respect to standard methods of AOAC[57] (AOAC 992.23), at room temperature, in triplicate. The protein content of each sample was calculated using Equation 3.1.

$$\text{Protein (mg)} = (\text{ml-blanc}) \times 0.1 \times 14 \times \text{KF} \qquad (3.1)$$

Where; 0.1 [M] is the HCl concentration that is used in titration; 14 [g/mol] is the molecular weight of nitrogen; KF is the Kjeldahl factor.

Protein characterization by SDS-PAGE

The protein profile of rapeseed was determined both for rapeseed flour and protein mixture. Samples de-fatted using Soxhlet apparatus were used to avoid excess oil.

The solid sample preparation was done according to Food Quality Department (Wageningen U&R) protocol. The required amount of sample was calculated according to the protein content percentage (1 mg protein/mL). Samples were mixed with 250 μl sample buffer (NuPAGE LSD Sample Buffer), as stated in the protocol, in 1.5 mL Eppendorf tubes (Greiner bio-one, Germany). For the reduced condition, samples were dissolved in the sample buffer with addition of 100 μl reducing agent (NuPAGE Sample Reducing Agents), and 650 μl of MiliQ water(Merk Millipore); for the non-reduced condition, only 750 μl of MiliQ water (Merk Millipore) was added, as stated in the protocol. Prepared mixtures were exposed to three freeze-thaw cycles. Freeze-thaw cycle was applied by placing the mixtures into

a freezer at -18 °C for 2 hours and then mixing for 10 min in a Thermomixer (Eppendorf AG, Germany) at 90 °C, 300 rpm. After three freeze-thaw cycles, the supernatant was recovered by centrifugation (VWR, Eppendorf AG, Germany), which performed for 1 min at 2,000 rpm, 23 °C. 12 μl sample and 10 μl protein marker (PageRuler Plus Prestained Protein Ladder 10-250 kDa, Thermo Scientific, Lithuania) were loaded into an electrophoresis gel (NuPAGE™ 4-12% BisTris Gel, Invitrogen, USA). The gel was placed in a gel tank (Life Technologies Mini Gel Tank, Thermo Scientific, The Netherlands) and run for 1 hour at 200 V, 45 mA using an electrophoresis power supply (VWR Power Source 300 V, USA). Then the gel was washed with demineralized water and stained with SimplyBlue™ SafeStain (Invitrogen™, USA). The gel was stored at room temperature under slow shaking, overnight (Innova® 44 Incubator Shaker Series, VWR, USA). After de-staining, gel was photographed with a Canon EOS 100D.

4.4. Preparation of Oil in Water Emulsion

Emulsion experiments were performed using the mixed protein mixtures, that were obtained by aqueous extraction method. Three different batches of protein mixtures were combined in order to guarantee an equal protein concentration of 1 g protein per sample. Oil in water emulsions were prepared as triplicate. The emulsions contained 10 g oil/100 mL and 0.5 g protein/100 mL.

First, the pH of the protein-water solution was adjusted to 7, using 0.5 M NaOH. The solution was stirred with a magnetic stirrer (VWR, USA) for 2 hours at minimum speed, for pH stabilization. Then, the solutions were pre-mixed with a high-performance dispersion instrument (Ultra Turrax®, IKA, Germany) at 8,000 rpm, for 1 min. Pre-mixed solutions were subjected to homogenization by a high-pressure homogeniser for 6 runs (Lab-Homogeniser, Delta Instruments, The Netherlands).

10 mL from each sample were stored in a fridge (4 °C) for 3 hours, which was required for emulsion stabilization. The stored samples were then used for size distribution and ζ–potential measurements. Remaining emulsion samples were divided into two portions. The first half was prepared for emulsion stability analysis and the other half was subjected to pH value change.

The pH value of the samples was adjusted to 3.8[67], by using 0.5 M HCl solution. Emulsions were stirred with a magnetic stirrer (VWR, USA) for 20 min at minimum speed, to stabilize the pH value. 10 mL from each sample were again stored in a fridge (4 °C) for size distribution and ζ–potential measurements. Remaining emulsion samples were prepared for emulsion stability analysis. The brief explanation of further analysis can be found in 4.5. Emulsion Characterization part.

4.5. Emulsion Characterization

Measurement of size distribution

In an emulsion, the size of the droplets have a strong effect on the stability[45]. Therefore, the size distribution of the emulsion is an indicator for further applications. To determine the size distribution of the emulsions, a laser light scattering instrument was used (Malvern Master Sizer 3000, Malvern Instruments Ltd, UK). The analysis was applied on fresh and aged (5 days) emulsions, at both pH values 3.8 and 7. The samples were analysed in triplicate at room temperature.

Before the analysis, the emulsions gathered from the fridge were stirred with a spatula for 2 minutes, to get a homogenous dispersion. Then, small aliquots were added into a mixing chamber, for which the obscuration rate was set at 5 to 15 %. Particle refractive index was set to 1.467[56] (for canola oil) and dispersant refractive index set to 1.33[56] (for water). The stirrer speed was set at 1200 rpm. The measurement cycle was repeated 5 times with a delay of 10 seconds. The measurements were

reported in respect to the surface weighted ($d_{32}= \sum n_i d_i^3/\sum n_i d_i^2$, n_i: number of droplets present) and volume weighted ($d_{43}= \sum n_i d_i^4/\sum n_i d_i^2$) mean diameter.

ζ–Potential measurement and titration curve

The electrical characteristics of a droplet surface in an emulsion, indicates the tendency to aggregate[33,45]. In general, to prevent aggregation, ionic emulsifiers are used to absorb the surface of the droplet[45,59]. To be able to indicate the electrical characteristics of a droplet surface, ζ–Potential measurement was done using a dynamic light scattering instrument (Malvern Zeta Sizer, Nano ZS, Malvern Instruments Ltd, UK). For the analysis, the emulsion was diluted with MiliQ water with a ratio of 1:100 (v/v). The reflective index was set for protein, at 1.450. The measurement took place at room temperature.

To determine the isoelectric point (pI) of the emulsion, a titration curve was plotted. For pH adjustment, an auto titration was used (Malvern Instruments Ltd, UK). The pH was adjusted using NaOH (0.5 M) and HCl (0.5 M) solutions. The pH range was between 2 to 12 with increments of 0.5. Measurement was done in 12 runs with 3 replications.

Emulsion stability against creaming

To assess the physical stability of the emulsions at pH values of 3.8 and 7, a visual observation was done. The observation took place at room temperature.

20 mL of each sample was placed in a capped cylindrical container. The height of the total emulsion and the serum that formed at the bottom of the container was measured with a ruler. The measurements were done at the 1st and the 5th day of storage.

Emulsion rheology

Rheological properties of the emulsions were determined by Rheometer Physica MCR 301 (Anton Paar 301, Korea) with controlled shear stress. Concentric cylinder geometry was used to characterize the low viscous emulsion (Measuring Cup, C-DG26.7/T200/Ti with internal and external diameter of 23.829 mm, and 27.600 mm, respectively; Measuring Cylinder, DG26.7/Ti with internal and external diameter of 24.651 mm, and 26.677 mm, respectively). During the analysis temperature was kept constant at 25 ^{o}C using a water pump (Water pump, Tetra, USA). RHEOPLUS/32 V3.62 was used as software. To determine the linear viscoelastic regime, the amplitude sweep determination was done at an oscillation frequency of 0.5 Hz. Viscoelastic properties of the emulsions were analysed at a constant deformation of 0.002 m, with frequency a range of 0.01 to 100 Hz. By changing the shear rate sweep with a range of 0.01 to 1000 Hz, viscosity profile was determined.

5. Results and Discussion

5.1. Composition and Characterization before the Aqueous Extraction Process

Before AEP, the black coated, edible rapeseed was ground to gain rapeseed flour for further investigation on composition and characterization. Moisture, oil and protein contents of this rapeseed flour were determined, and extraction yield was calculated.

The determined composition of rapeseed flour was found to have a similar protein and oil content compared to literature (Table 5.1). Protein was measured using Kjeldahl method. The Kjeldahl method measures the total nitrogen content, therefore, it can give a false conclusion. For instance, in this study the de-hulling step was skipped. Therefore, apart from protein, non-protein components in the hulls were also measured during the analysis[29]. Both conversion factors 6.25 and 5.7 were used for calculation of protein. The conversion factor 6.25 is a commonly used factor to convert nitrogen to crude protein, and was used to compare the results with the current literature[12,33,46,47]. Factor 5.7, used for oil seed proteins[46].

The comparison of the results with literature data (Table 5.1) show a difference in oil content, which can be accepted, since oil content varies with the seed type[12,28,33] and the color of the seed coat. Black seed coated rapeseed has a lower oil content compared to yellow seed coated[33].

Table 5.1. Determined Composition of Rapeseed Flour

Composition	Rapeseed Flour (dm %)	Average Composition from Literature* (%)
Protein (N*6.25)	18.66 ± 1.77	21
Protein (N*5.7)	17.02 ± 1.62	
Oil	37.95 ± 0.49	43

**See Table 3.1. in the Background*

ζ-Potential and Titration Curve

To obtain the major protein fractions namely, napin and cruciferin, precipitation at isoelectric point was applied. By changing the pH towards a value where the proteins have net zero charge (isoelectric point, pI), the repulsive forces between the proteins diminish and aggregates are formed[58]. Each protein in the mixture can have different isoelectric points. Therefore, the pI of the dissolved rapeseed flour was determined to be able to precipitate all rapeseed proteins. The pI value was found to be around pH 5 (Figure 5.1). At acidic pH values, between 2 to 4.8, the surface charge of rapeseed proteins was positive whereas at slightly acidic and basic pH values, between 5 to 12, the surface was negatively charged. Besides, the degree of electrostatic repulsion between adjacent particles could be determined from the Figure 5.1. At high ζ-potential values (either positive or negative), which were between pH 2 to 3 and 10 to 12, resistance to aggregation[32,58] is high. On the contrary, at low zeta potential values, which were between pH 4 to 7, the tendency is aggregation, since the attractive forces between the adjacent particles may exceed repulsive forces[32,58].

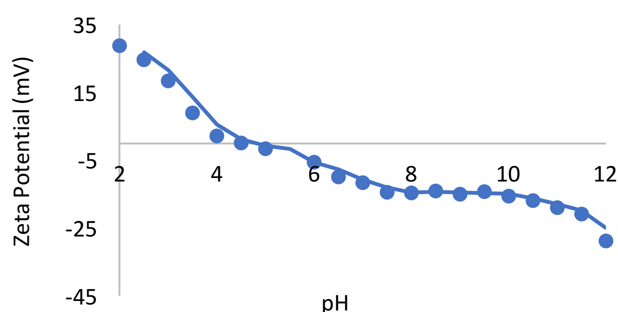


Figure 5.1. Titration curve of the rapeseed protein

The isoelectric-point also confirmed by studies, which determined the lowest solubility of the rapeseed protein at pH values between 3 to 5 due to the net zero charge of the rapeseed proteins[12,19,32,53,55].

5.2. Composition and Characterization after the Aqueous Extraction Process

After the aqueous extraction process, freeze dried protein mixture was subjected to measurements for determination of composition. The results showed that a high amount of protein, significant amount of oil and a minor amount of the rest - biomass were obtained as product (Table 5.2). The obtained results considering composition and extraction yield were compared with literature. However, since there are not published studies about aqueous rapeseed protein extraction, the conventional rapeseed protein extraction route and AEP for soybean were taken into consideration. The rest of the biomass was expected to contain carbohydrates, lignin and impurities as stated in the literature (Table 3.1). However, the components other than oil and protein were not of concern of this thesis.

Table 5.2. Determined Composition of Protein Mixture

Composition	Protein Mixture (dm %)
Protein (N*6.25)	65.46 ± 6.46
Protein (N*5.7)	59.70 ± 5.02
Oil	16.90 ± 0.88

After determination of the protein content, protein yield of the process and the extraction yield were calculated using equations 5.1, 5.2 and 5.3. Results were tabulated on Table 5.3. Protein content and extraction yield depend on the pH of extraction media, agitation rate, solid to water ratio, particle size, extraction time, number of extraction stages and temperature of the extraction media[48,50,53,54,55]. Therefore, to obtain high purity and high extraction yield, more research should be done in properties of rapeseed proteins and optimal extraction conditions.

$$\text{Protein yield (dm \%)}=\frac{\text{Protein \% (N*6.25)} \times \text{Freeze Dried Product (g)}}{\text{Protein \% (N*6.25)} \times \text{Dried Rapeseed Flour (g)}} \times 100 \quad (5.1)$$

$$\text{Extraction yield (\%)}=\frac{\text{Collected Pellet (g)}}{\text{Rapeseed Flour (g)}} \times 100 \quad (5.2)$$

$$\text{Extraction yield (dm \%)}=\frac{\text{Freeze Dried Product (g)}}{\text{Dried Rapeseed Flour (g)}} \times 100 \quad (5.3)$$

Table 5.3. Yield of the Aqueous Extraction Process

Protein yield (dm %)	Extraction yield (%)	Extraction yield (dm %)
12.55 ± 2.53	15.95 ± 6.81	4.00 ± 1.37

In general, the conventional rapeseed protein extraction route has a protein content range between 70 % to 90 %[50,53,54,55]. The content depends on selected unit operations and conditions. For instance, Ghodsvaly et al.[55] obtained 85% protein purity, with extraction yield of 15 % and protein yields of 30 %. This result was similar to our findings in respect to extraction yield but the extraction route should be examined in depth.

To solubilize the rapeseed protein in the aqueous media, the extraction was carried out distant from the iso-electric point to prevent precipitation the proteins. In this thesis pH 8 was chosen, that was distant from isoelectric-point of each major protein fraction, which also claimed to be the general reference pH for protein extractions[50,51]. Several studies show that increasing pH value in alkaline media can increase the extraction yield. Rosenthal et al.[50] and Campbell et al.[51] both reported that, the extraction yield had a minor change from pH 8 to 10 (approx. 80 % yield) and had distinguishable increase after pH 10 (approx. 95 % yield). As it can also be seen in Figure 5.1, the protein solution had approximately same zeta potential value from pH 8 to 10. Above pH 10, the zeta potential value increased which also indicates to slight changes of precipitation. However, protein hydrolysis is likely above pH 10[32].

In respect to the extractability of proteins, pH of the alkaline media is also correlated with the protein concentration in the alkaline media[32,48,50]. Gerzhova et al.[32] highlighted that the extractability increases with increasing concentration, and in low pH values the extractability is affected more with increasing concentration than in high pH values. Depending on the selected pH 8, solid to water ratio was chosen as 1:7 (concentration: 12,5%). To determine the optimal solid to water ratio more studies should be done.

Protein losses could occur at possible bottlenecks like grinding, filtration and centrifugation steps. In the grinding step, the power could not be sufficient to release all the nutritional components out of the seeds and hulls or cotyledon. In the filtration step, the retentate was mostly consisted of hulls that are also a protein reserve. In the centrifugation step, three streams were created, namely: an oil cream fraction containing stabilized-OBs, an aqueous phase with dissolved proteins, and a small residual solid phase that was expected to contain primarily cellulosic and hemicellulosic materials from the hulls. As mentioned before, due to pH of the extraction media, proteins interact with each other and form aggregates which results in precipitation. Therefore, it is possible that there was a protein loss in the centrifugation step where some proteins aggregate and merge in the pellet and could not be obtained at subnatant part. Also, oil in the protein mixture could be explained by the same mechanism. During the experiments the cream layer that contained oil was scoped. However, due to the composition, it was possible that protein interacted with oil. By surrounding around oil particles, the protein might have prevented oil particles to move upwards due to higher density and drew them into subnatant phase or into pellet[65]. Besides, several centrifugation steps could make them coalescence and precipitate. On the other hand, the significant amount of oil content was relevant to the omitted de-fatting step.

Moreover, due to high mobility of small particles in disrupted cells, small particle size accompanies high yield on protein and oil extraction[50,51]. By grinding, blending and stirring, high shear forces help to release more nutritional compounds but also help to form more stable emulsions depending on the oil droplet size. This argument will be discussed in detail in the 5.3. Emulsion Stability.

Temperature is another parameter effecting the yield. A temperature range between 20 to 30 °C is suitable for the extraction[52]. Temperatures above 30 °C increase the solid yield, whereas reveal protein denaturation and decrease protein and oil extraction yield[50,63]. In this thesis, extraction was performed at room temperature. This could be one of the reasons of low extraction yield (see Table 5.3).

During the pH adjustment for extraction media, an antagonistic buffering effect was observed due to rapid protein and oil release. To obtain all the nutritional compounds into the media, stabilization had to be achieved. Campbel and Glatz [51] stated that the yield remained constant after 120 min of extraction. It is however, depending on several parameters such as solid to liquid ratio and temperature.

To increase the recovery rate of products certain process steps could be repeated, like increasing of the number of extractions by the aqueous extraction process. As Nikiforidis and Kiosseogluo[49] stated, by increasing number of extractions from 1 to 3, extraction yield increases in a range of 20 % to 60 % depending on pH of the extraction media and size of the raw material.

SDS-PAGE

To estimate the recovery of the rapeseed protein fractions, the protein profiles of rapeseed flour and protein mixture were compared; reducing and non-reducing conditions were applied (Figure 5.2). The results for rapeseed protein profile was coherent with literature data[12,24,29,30,42].

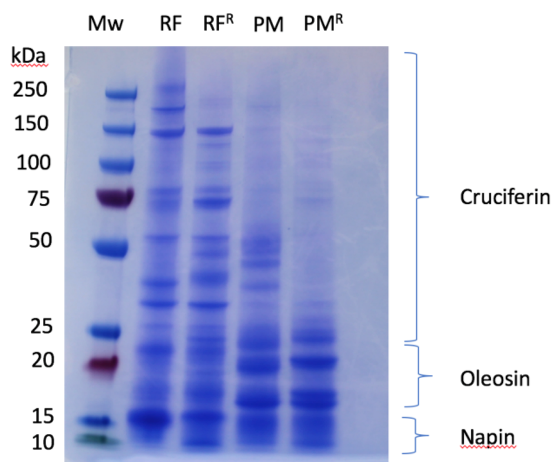


Figure 5.2. SDS-PAGE of rapeseed protein. Mw: Protein molecular weight marker, RF: Rapeseed flour, RF^R: Rapeseed flour in reduced condition, PM: Protein mixture, PM^R: Protein mixture in reduced condition. The protein mixture was obtained at the isoelectric point of the rapeseed protein (approx. at pH 5)

In the RF column, there were 4 major bands with high intensity, 150, 30, 23, 15 kDa, which represented crucifern, oleosin and napin (Figure 5.2)[24,29,49]. In the presence of a reducing agent (Figure 5.2, column RF^R) high molecular weighted polypeptides at 300 and 200 kDa disappeared and then dissociated into lower-molecular weighted polypeptides, which indicated the presence of disulphide bonds[32,12,19,29,53,54] (Figure 5.2, column RF, RF^R). Besides, in column RF^R low contrast bands were also observed within a line. This phenomenon might be either due to proteins with low purity or it was the sign of further protein degradation (see in Fig. 5.2 the appearance of double bands at around 50, 23 and 15 kDa).

In the PM column, four major bands were determined with molecular weights of 16, 18, 30 and 53 kDa, as also stated by Aluko and McIntosh[53], that identified the 3 major protein fractions in rapeseed (Figure 5.2). Compared to the RF column, it could be seen that bands identified as cruciferin were either disappeared or had a slight intensity (Figure 5.2). This result could be a proof that cruciferin was

strongly affected by the process conditions and the removal of the fraction was incomplete. Cruciferin is a salt soluble protein[29], whereas in this thesis salt was not added into the extraction media. Therefore, it was possible that cruciferin was not soluble enough in the extraction media. This hypothesis was confirmed by Gerzhova et al.[32] that high molecular weighted polypeptides were observed after the salt addition. Another aspect is that, pure cruciferin has an isoelectric point at pH 7.25. This value is close to pH of the extraction media, which could cause unwanted precipitations during extraction. Protein loss could have been occurred in the centrifugation step. In the PM^R column, the two major bands from PM column at 50 and 29.5 kDa and a minor band at 44 kDa disappeared, which is also observed by Wu and Muir[64]. This is an indication of presence of disulfide linkage of cruciferin.

Napin fraction was identified in both rapeseed flour and protein mixture, where the bands were at 8 and 15 kDa. The intensity of the napin bands were lower in the PM column compared to RF column (Figure 5.2). The pI of napin is at pH 11 and it is expected that napin can solubilize at lower pH values. Since the isoelectric precipitation was applied at around pH 5, it was possible that some of napin was solubilized in aqueous media and could not be precipitated and could not be obtained in the pellet. This hypothesis is supported by Wanasundara et al.[29]: SDP page show for extraction conditions at low acidic pH, such as 2 and 4, only bands of napin (at 8 to 15 kDa) and at pH values 7 and 10, bands of cruciferin and napin (at 8, 34 and 50 kDa) are observed. The bands at 8 and 34 kDa can also be seen in Figure 5.2. In the column PM^R, lower molecular weighted polypeptides at around 8 kDa were formed compared to PM column. Gerzova et al.[32] found similar results, which highlights the disulfide linking of napin.

Oleosin was detected at 16 and 18 kDa[65,66]. The bands were more intense at columns for rapeseed mixture than in rapeseed flour. Oleosins are membrane proteins which are also referred as oil body proteins (OBP)[12,24]. According to Nikiforidis et al.[66] oleosin was seen in the cream that consists of oil bodies (OB). Therefore, the presence of oleosin could be related to the oil content in the extraction mixture. Oleosin bands became more intense in the PM^R, than in PM, which could be because of the new polypeptide formation instead of disulfide linking[32].

5.3. Emulsion Stability

Food emulsions are micro-heterogenous materials they differ in size, shape and physicochemical properties. The characteristics of droplets dominate the physicochemical properties of the emulsion. For emulsion stability, droplet size, droplet charge, interfacial properties and colloidal interactions between the droplets determine the stability of an emulsion. Dairy products are stable at pH7 and salad dressing type food products are stable at pH 3.8[67]. In this thesis stability of emulsions at pH 7 and 3.8 were compared, in order to estimate the suitable market for the rapeseed mixture that was obtained by AEP.

Size Distribution

In general, food emulsions contain a variety of droplet size[45]. Therefore, this polydisperse system is characterized in respect to the concentration of droplets in different size classes (particle size distribution). In the analysis, the particle size was specified with the diameter. Two mean particle size values were used by the determination of the distribution: surface weighted mean diameter (d_{32}) and volume weighted mean diameter (d_{43}). d_{32} is more sensitive to individual and the majority of particles; d_{43} is more sensitive to large particles and the few large aggregates[45]. A typical mean droplet diameter of an emulsion is approximately 1 µm, which can vary with emulsification conditions and emulsifier concentration[59].

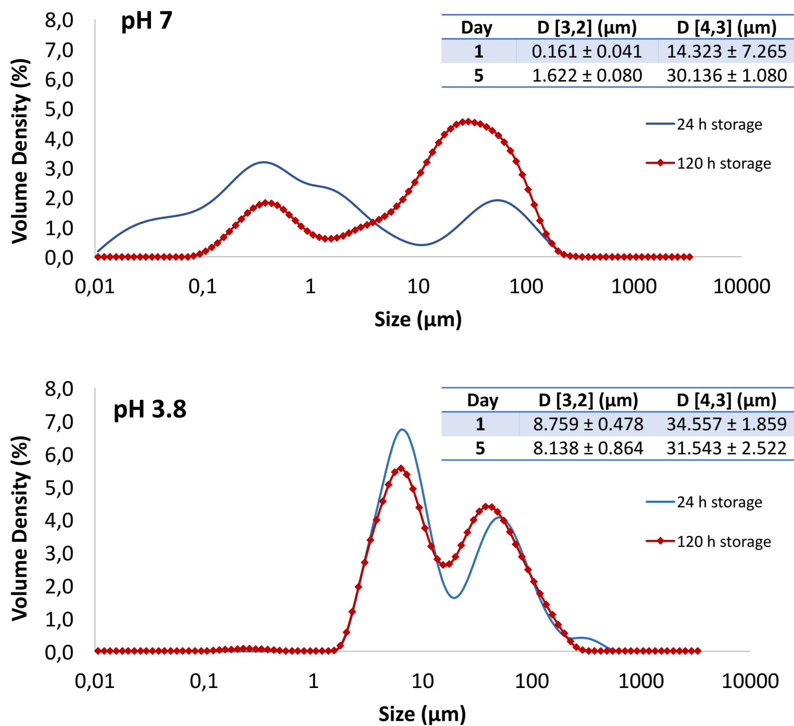
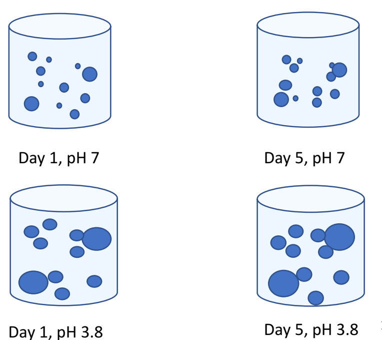


Figure 5.3. Droplet size distribution of 0.5 % rapeseed protein mixture emulsion. a: Emulsion at pH 7, b: Emulsion at pH 3.8. The emulsions stored at 4 °C for total of 5 days.

The size of the droplet was plotted against the volume density (%) (Figure 5.3 a,b). Two major peaks were observed in both pH values and storage period (Figure 5.3. a, b), thus, emulsions having a bimodal distribution. After 1 day of storage, emulsion at pH 7 had a smaller individual droplet size (0.161 µm) with a high distribution and small aggregations. On the other hand, emulsion at pH 3.8 had a bigger individual droplet size (8.759 µm) with a narrow distribution and more aggregations compared to emulsion at pH 7. Compared to the generalized emulsion droplet size, the droplet size at pH 3.8 was too large. For this specific pH value, the emulsification conditions and/or concentration of the emulsifier (protein mixture) might not be compatible[59]. After 5 days of storage, for the emulsion at pH 7, the size of the individual droplets was shifted to larger droplets and the aggregation increased (Figure 5.3.a). For the emulsion at pH 3.8 there was not a remarkable change but the size distribution was shifted to right, larger size range (Figure 5.3.b). Shifting to higher diameters is a usual phenomenon for emulsions after several days of storage that indicates the destabilization process[70]. A similar trend for size distribution under storage period was obtained by Nikiforidis and Kiosseogluo[49], who used maize germ as emulsifier. Other authors have also reported the shift of size distribution and investigated the shift at different pH values (Mantovani et al[70]).



5.4. Schematic representation of droplet size change in the emulsion, based on Figure 5.3

These results were expected, considering the titration curve for the emulsion with rapeseed protein mixture (Figure 5.4). Droplet charge in emulsions is an important parameter for droplet stability and physicochemical properties. It also effects the interaction with other charged species like ions in water etc. According to the Figure 5.5, repulsive forces between droplets diminish at the iso-electric point and cause droplets cluster and form aggregates. Thus, at iso-electric point (pH 5), the emulsion system had the least stability. The results about the size distribution were compatible with the titration curve. At pH values 4 to 6 the zeta potential value was remarkably low which means that aggregation was likely to occur. Since pH 3.8 is close to the iso-electric point, it was expected to have aggregation in parallel with big droplet size. In addition, the droplets at pH values between 2 to 4 and 6 to 12 had sufficiently negative or positive charges to form a more stable emulsion. At those pH values, the repulsive forces between adjacent proteins dominate over the attractive forces and the droplets are less likely to cluster and form aggregates. As indicated in Figure 5.5, pH 7 was further away from the iso-electric point compared to pH 3.8 that it was expected to have less aggregation. Besides, since proteins were used as emulsifier, hydrophobic interactions might occur even though, repulsion is the net result due to the corporation of steric and electrostatic repulsion. When the pH is closer to the pI of protein, electrostatic attraction occurs between negative and positive groups on the surface. At pI, hydrophobic attraction occurs and the emulsion droplets tend to aggregate (Figure 5.5)[59].

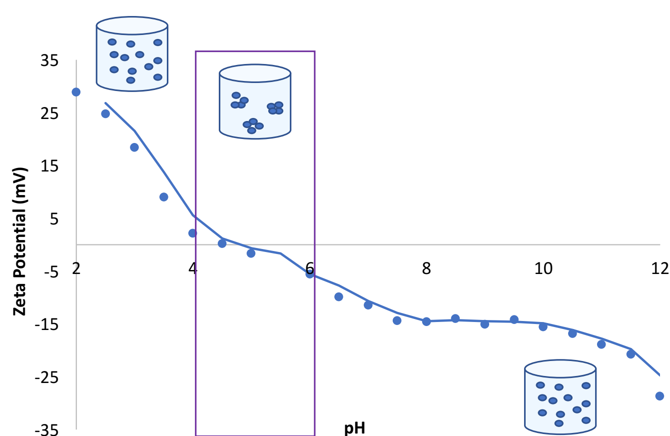


Figure 5.5. Titration curve of 0.5 % rapeseed protein mixture emulsion. pH values range between 2 to 12, with increment of 0.5.

Creaming Behaviour

In general, the density of the droplets in an emulsion is different from the liquid that surrounds them. In the oil in water emulsion (o/w) systems, oil has a lower density than the aqueous phase. When the emulsion is stored for further use, the net gravitational force acts upon oil droplets. Due to density difference, oil droplets start to move to the top of the emulsion. This instability phenomenon is called creaming. Creaming behaviour is an important parameter for shelf-life of the emulsion and for further applications. The rate of the creaming can be determined by Stokes Law for an isolated spherical particle in an ideal (Newtonian) liquid (Equation 5.4)[45]. According to Stokes law, creaming behaviour is affected by the droplet size, density of dispersed and continuous phases and the viscosity of the continuous phase.

$$v_{Stokes}=-\frac{2gr^2(\rho_2-\rho_1)}{9\eta_1} \quad (5.4)$$

Where, v_{Stokes}: creaming velocity, r: radius of the droplet, g: acceleration due to gravity, ρ: density, η: shear viscosity, 1: continuous phase, 2: dispersed phase.

The creaming behavior was measured for emulsions at pH 7 and pH 3.8. As shown in Figure 5.6, after 1 day of storage at pH 7 there was no indication of creaming. However, at pH 3.8, creaming had already occurred. After 5 days of storage at both pH 7 and 3.8 a serum layer occurred at the bottom of the emulsion in each sample. The serum layer at pH 3.8 and after 5 days of storage was clearer, which means that the cream layer was packed more closely on the top of the emulsion.

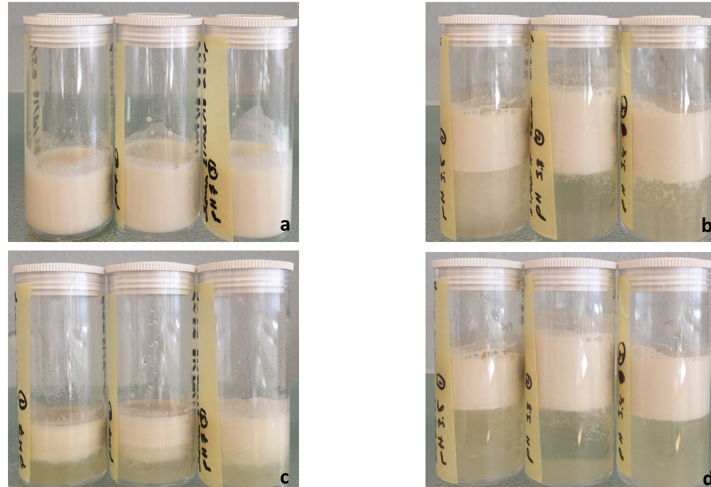


Figure 5.6. Visual observation for creaming behaviour. a-b: 0.5 % rapeseed protein emulsion after 1 day of storage, c-d: 0.5 % rapeseed protein emulsion after 5 days of storage. Where, samples a and c were having pH value of 7 and samples b and d were having pH value of 3.8.

To get a better understanding about the rate of the creaming, the height (ratio of serum to total emulsion) versus time was plotted (Figure 5.6). The result shows that there was a rapid creaming at pH 3.8 and a slight increase after 5 days. On the other hand, emulsion at pH 7 had less creaming where, it was stable after 1 day of storage and then a remarkable increase (Figure 5.7).

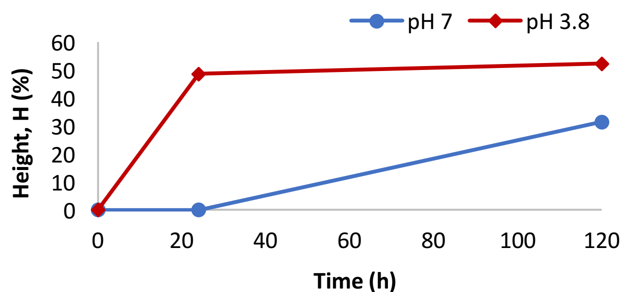


Figure 5.7. Creaming behaviour of 0.5 % rapeseed protein emulsion. The emulsions stored at room temperature for total of 5 days. Height is the ratio of serum to total emulsion.

It is known that rapid creaming is a result of aggregation[45,59]. The aggregation formation is depended on the colloidal interactions between emulsion droplets, which determine the physical stability of the particles. The interaction types can be grouped as repulsive (electrostatic, steric) and attractive (van der Waals, hydrophobic, depletion)[59] forces. Attractive interaction causes the tendency to aggregate and repulsive interaction provides droplets to be individual[59].

The reason of the fast creaming behaviour at pH 3.8 was due to size distribution and the droplet charge; bigger droplets were more likely to cluster (Figure 5.3, b). Due to the pH value, attractive colloidal interactions dominated, which caused the aggregation. After 5 days, the droplet size grew larger and the aggregation increased (Figure 5.3, b). This was the explanation of the more packed cream layer after 5 days of storage (Figure 5.6). At pH 7, at the first day of storage, individual droplets were small and consequently more stable (Figure 5.3, a). This could be related to the non-existence of cream layer in the first day of storage (Figure 5.6). After 5 days of storage, at pH 7, there was a remarkable increase in creaming behaviour. The reason could be that at day 5 the individual droplet size grew larger and more aggregations were formed (Figure 5.3, a). The color of the emulsion is also an indicator about the size of the droplets. Emulsions tend to be white when the droplet size is around 3 µm and by increasing size up to 30 µm, the color changes to yellow (color of oil) [59].

These results indicated that small droplets made the emulsion more stable in respect to creaming behaviour. Formation of aggregates with small droplets was less likely and the creaming slow in comparison with big droplets. Small size droplets needed more time to get out of the dispersed phase and to move at the top of the emulsion. Nikiforidis and Kiosseogluo[65] also stated that one of the reasons for a lower stability might due to the larger mean droplet size which was expected to have rapid creaming.

Rheological Characterization

Rheological characterization about emulsions give some information about (micro) structure of the system. The characterization measurements were carried on only for the emulsion at pH 7, because it was more stable than the emulsion at pH 3.8.

Firstly, amplitude sweep and frequency sweep measurements were applied to indicate whether the emulsion system is having a liquid-like or gel-like structure. It is an important parameter for storage and further applications.

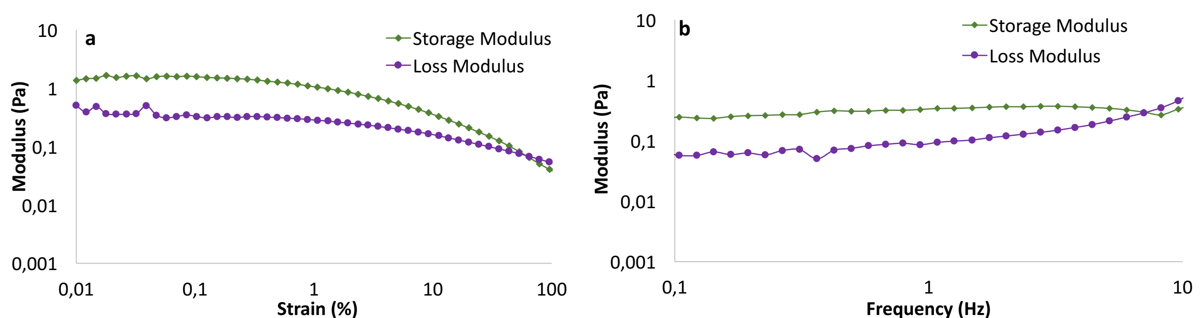


Figure 5.8. a: Amplitude sweep determination at a constant oscillation frequency of 0.5 Hz; b: Frequency sweep determination at a constant deformation of 0.002, with frequency range of 0.01 to 100 Hz. The measurements were done at room temperature.

Two parameters were recorded during the measurement to estimate the viscoelastic response: storage modulus (G') and loss modulus (G''), where G' gives information about elastic response and G'' is about viscous response[61].

Amplitude sweep gives the rigidity and strength characteristics of the emulsion. Besides, with the amplitude sweep the linear viscoelastic region (LVER) is also determined. This region is important to

determine the stability. In this measurement, LVER observed from 0,01 to 1 % strain (Figure 5.8, a). The longer the region is, better the possibility of a stabile system[62].

At low stress, the emulsion preserved its structure but around 90 % strain, disruption of the structure occurred (Figure 5.8, a). Till reaching 90 % strain, the storage modulus (G') is larger than the loss modulus (G''), which means the applied force is still smaller than the inter molecular forces between the emulsion droplets. In this case, the material acts as an elastic solid, where the stored energy can still be returned without structure deformation. However, when the force is higher than the inter molecular forces, G'' becomes larger than G'. When the storage modulus (G') is larger than the loss modulus (G''), which means the applied force is still smaller than the inter molecular forces between emulsion droplets. In this case material acts as a weak elastic solid, where the stored energy can still be returned without structure deformation (network/gel-like structure). However, when the force is higher than the inter molecular forces, G'' becomes larger than G'. That means, the network-like structure disrupted and become inline, which means that material started to flow. In our case, after 90 % the emulsion structure disrupted and the given energy start to dispersed (Figure 5.8, a).

Frequency sweep shows the structural response of the emulsion to deformations in longer or shorter timesclaes[60]. It is an important parameter to predict the behaviour of the emulsion at storage and application conditions. In Figure 5.8, b, emulsion was exposed to deformation oscillations. The frequency sweep force was kept constant and the frequency increased (Figure 5.8, b). When the frequency increased, the deformation was faster. A similar result regarding the frequency sweep was obtained by Nikforidis et al.[67], where emulsions were made of oil bodies and egg yolk blends. In solid particles, droplet interactions are higher, which means less frequency. It was observed that approximately up to 9 Hz, emulsion acted elastic and after that the structure became viscous (Figure 5.8, b). In that case, the system seemed to act as gel and creaming was unlikely. This hypothesis was confirmed by visual observation for creaming behaviour (Figure 5.6, a). Creaming was not observed in 24 hours, signified that the emulsion had a gel like structure and rigid network.

Secondly, viscosity profile of the emulsion was analysed (Figure 5.9). Emulsions are referred as non-Newtonian fluid. The viscosity of the emulsions varies depending on shear rate that they are exposed to (apparent viscosity)[60]. Non-Newtonian fluids tend to decrease with increasing shear rate[59,60]. This phenomenon is called shear-thinning. In general, most systems come across to shear thinning[59,65,67,68]. The observed viscosity at that point is the apparent viscosity. For Newtonian fluids, apparent viscosity is constant; for non-Newtonian fluids, it is depending on the shear rate[59]. Shear thinning profile started at a viscosity of 5 Pa.s with a shear rate of 0.012 s^{-1} and decreased to 0.018 Pa.s with a shear rate of 1000 s^{-1}. When shear was applied, the droplets that had a network-like structure became in line, which means that they could flow easily. Therefore, viscosity was decreased. These results are compatible with White et al[69], who assessed the rheology and stability of emulsion which formed by sunflower seed oil bodies. The point at around 5 Pa.s, is called zero-shear viscosity[60]. This point is inversely proportional to creaming velocity of emulsion droplets, thus an important indicator for the emulsion stability[60].

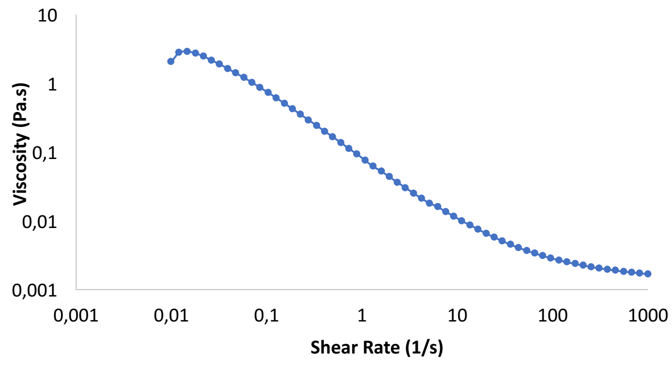


Figure 5.9. Viscosity versus shear rate diagram for 0.5 % rapeseed protein emulsion after 24 h of storage, at 4 ^{o}C

6. CONCLUSION

In this thesis, an insight into rapeseed protein fractions was gained and the ability e of the aqueously extracted protein mixture to stabilise emulsions was investigated. The current literature does not describe any aqueous extraction process for rapeseed. Our non-optimized process yielded a protein mixture with low purity and yield. Better results for yield and purity could be obtained by optimization of process parameters like particle size of the raw material, pH of the extraction media, agitation rate, concentration of the media, duration and number of extraction stages and temperature of the media. Moreover, protein characterization showed that all the major rapeseed protein fractions were obtained whereas, cruciferin was strongly affected by the process.

Emulsions, produced using the obtained protein mixture, had different structures and characteristics at pH 3.8 and 7. At pH 7, the individual size of the droplets in the emulsion was smaller than at pH 3.8 and the aggregations were lower. The effect of the interactions between the droplets directed the stability of the emulsion at storage period. After 5 days of storage, the size distribution shifted to higher diameter range at both pH values. Combining the size distribution results with creaming behaviour indicated that small droplet sized emulsions were less likely to from aggregates and the creaming rate was smaller than in large droplet sized emulsions. The emulsion structure at pH 7 was found to depend on frequency; it was gel-like at low and became more viscous at high frequencies. The viscosity of the emulsion decreased by increasing shear rate. The structural characterization of the emulsion indicated that droplets had interactions with each other and the emulsion could be disrupted by application of force.

These results proved that rapeseed protein mixture could be an alternative for food applications, especially for dairy industry.

7. RECOMMENDATIONS

The aqueous extraction process should be optimized to increase the extraction yield and protein purity. Here are some promptings for further studies at different process steps:

- De-hulling: in this study de-hulling was excluded. To have higher yields, de-hulling could be applied.
- Centrifugation and filtration: Characterization of protein and determination of protein and oil contents, should be done after centrifugation and filtration steps. Protein losses, solubility of each fraction, effect of the media-pH on each protein fraction could be determined.
  During centrifugation, the cream layer was scoped and was not used or analyzed in this study. After each centrifugation step, the cream layer should be analyzed. Composition of the protein mixture and protein profile would give more insight into rapeseed protein fractions. Knowledge about solubility of different protein fractions could lead to the reason of losses.
- Extraction: extraction could be repeated at least 3 times for higher yields.
  Salt was not added into extraction medium in this study. However, cruciferin recovery was not high enough. Salt addition could be an option to increase cruciferin recovery.
- Emulsion: The concentrations of each protein fraction in the protein mixture should be determined in order to understand the effect of each fraction in the emulsification process. Also, the concentration of the protein mixture in the emulsion should be optimized.
  Protein mixtures provide a better stability to emulsions. More research should be done about the mechanisms of stabilization.
  To get smaller droplets thus, more stable emulsions, pre-mixing and homogenization could be run in higher agitation rate and pressure. This effect should be investigated relating to other parameters like pH.
  To understand more about changes in size distribution, microscopic examination could be applied to identify whether the increase in size is due to aging or coalescence.
  Microscopic examination could also help to determine the mechanism between the oil inside the rapeseed protein mixture with the added oil to find out whether they cluster together or not.
- Food applications, studies should be done according to emulsion formation at different pH values.

REFERENCES

[1] Global Rapeseed Production. (2017). Retrieved September 10, 2017, from http://www.globalrapeseedproduction.com/

[2] OECD & FAO. (2015). Agricultural outlook 2015-2024. Retrieved September, 10, 2017, from www.Fao.Org/3/a-i4738e.Pdf

[3] Haar, D.V., Müller, K., Bader-Mittermaier, S., & Eisner, P. (2014). Rapeseed proteins-Production methods and possible application ranges. *Ocl, 21(1).* doi:10.1051/ocl/2013038

[4] Heuze V., Tran G., Sauvan D., Lessire m., and Lebas F., (2017). Rapeseed meal. Feedipedia a program by INRA, CIRAD, AFZ and FAO. https://feedpedia.org/node/52. Last updated on June 21, 2017.

[5] Campbell L., Rempel C.B. & Wanasundara J.P.D. (2016). Canola/Rapeseed Protein: Future Opportunities and Directions-Workshop Proceedings of IRC 2015. Plants (Basel). *2016 Apr 13; 5(2).* doi: 10.3390/plants5020017

[6] Schweizer M., Segall K., Medina S., Willardsen R., Tergese J. (2006). Rapeseed/canola protein isolates for use in the food industry. *Process Technology.*

[7] Nikiforidis C. Protein complex mixtures for food emulsions. (2016)

[8] Perera S. P., McIntosh T.C. and Wanasundara J.P.D. (2016). Structural Properties of Cruciferin and Napin of Brassica napus (Canola) Show distinct Responses to Changes in pH and Temperature. *Plants (Basel*). 2016 Sep 7; 5(3). doi: 10.3390/plants5030036

[9] Fisher, E. (2015). Alternative Proteins to Claim a Third of the Market by 2054. Retrieved September 20, 2017, from http://www.luxresearchinc.com/news-and-events/press-releases/read/alternative-proteins-claim-third-market-2054

[10] Billing, S. (2016). Protein Challenge 2040: Sustainable protein production for the future. Retrieved January 10, 2018, from https://thefuturescentre.org/articles/8213/protein-challenge-2040-sustainable-protein-production-future

[11] Henchion, M., Hayes, M., Mullen, A., Fenelon, M., & Tiwari, B. (2017). Future Protein Supply and Demand Strategies and Factors Influencing a Sustainable Equilibrium. *Foods, 6(12),* 53. doi: 10.3390/foods6070053

[12] Aider M. & Barbana C. (2011). Canola proteins: Composition, extraction, functional properties, bioactivity, applications as a food ingredient and allergenicity - A practical and critical review.*Trends in Food Science & Technology, 22(1)*, 21-39. doi: 10.1016/j.tifs.2010.11.002

[13] Eriksson, I., Westerlund, E. & Aman, P. (1994). Chemical composition in varieties of rapeseed and turnip rapeseed, including several samples of hull and dehulled seed. *J. Sci. Food Agric. 66 (2)*, 233–240. doi: 10.1002/jsfa.2740660219

[14] Henchion, M., Hayes, M., Mullen, A., Fenelon, M., & Tiwari, B. (2017). Future Protein Supply and Demand: Strategies and Factors Influencing a Sustainable Equilibrium. *Foods*, *6(12)*, 53. doi: 10.3390/foods6070053

[15] United States Department of Agriculture, World Agricultural Outlook Board, Foreign Agricultural Service, economic Research Service, & Agricultural Marketing Service. (2017). World Rapeseed and Products Supply and Distribution. Retrieved October 12, 2017 from https://apps.fas.usda.gov/psdonline/reportHandler.ashx?fileName=Table17:World:RapeseedandProductsSupplandDistribution&reportId=716&templateId=13&format=html

[16] Food and Agriculture Organization of the United Nations (2017). Crops. Retrieved, October 12, 2017, from http://www.fao.org/faostat/en/#data/QC/visualize

[17] Gustone, F. D. (2004). *Rapeseed and canola oil: Production, processing, properties and uses.* Oxford, EN: Blacwell Pub.

[18] Çulcuoğlu E., Ünay, E., & Karaosmanoğlu, F. (2002). Rapeseed Cake as Biomass Source. *Energy Sources, 24:4*, 329-336, dOI: 10.1080/00908310252888709

[19] Tan, S.H., Mailer, R. J., Blanchard C.L., & Agboola S. O. (2010). Canola Proteins for Human Consumption: Extraction, Profile, and Functional Properties. *Journal of Food Science, 76(1).* doi: 10.1111/j.1750-3841.2010.01930.x

[20] AarhusKar Ishamn. (2017). Rapeseed Oil. Retrieved October 23, 2017, from http://www.aak.com/Global/Brochures/Rapeseedoil_from_AAK.pdf

[21] Agricultural Marketing Resource Center. (2017). Rapeseed. Retrieved October 23, 2017, from https://www.agmrc.org/commodities-products/grains-oilseeds/rapeseed/

[22] Dworakowska S., Bednarz, S., & Bodgal, D. (2011). Production of biodiesel from rapeseed oil. 1st World Sustainability Forum (1-30 November 2011)

[23] Herkes, J. (2014). Rapeseed and Canola for Biodiesel Production. Retrieved November 4, 2017, from http://articles.extension.org/pages/26629/rapeseed-and-canola-for-biodiesel-production

[24] Wanasundara, J.P.D., McIntosh, T.C., Perera, S.P., Withana-Gamage, T. S., & Mitra, P. (2016). Canola/rapeseed protein-functionality and nutrition. *Ocl,23(4).* doi: 10.1051/ocl/2016028

[25] National Research Council (US) Subcommittee on the Tenth Edition of the Recommended Dietary Allowances. Recommended Dietary Allowances: 10th Edition. Washington (DC): National Academies Press (US); 1989. Available from: https://www.ncbi.nlm.nih.gov/books/NBK234932/doi: 10.17226/1349

[26] Zayas, J.F. (1997). *Functionality of proteins in food.* Berlin: Springer

[27] Johnson, D.L., & Croissant, R.L. (1992). Rapeseed/Canola Production. *no.0110.* Retrieved October 23, 2017, from https://dspace.library.colostate.edu/bitstream/handle/10217/183517/AEXT_ucsu2062201101992.pdf?sequence=1

[28] Rad, A.H.S., and Zandi, P. (2012). Comparison of Winter and Spring Rapeseed Cultivars Considering their Oil Content and Fatty acids composition. *American-Eurasian J. Agric. & Environ. Sci., 12 (2):* 243-248.

[29] Wanasundara, J.P.D., Abeysekara, S.J., McIntosh, T.C., & Falk, K.C. (2011). Solubility Differences of Major Storage Proteins of Brassicaceae Oilseeds. *Journal of the American Oil Chemists Society, 89(5),* 869-881. doi: 10.1007/s11746-011-1975-9.

[30] Wanasundra J.P.D. (2011). Proteins of Brassicaceae Oilseeds and their Potential as a Plant Protein Source. Critical Reviews in Food Science and nutrition, 57(7), 635-677. doi: 10.1080/10408391003749942

[31] Khattab, R., Eskin, M., Aliani, M. & Thiyam U. (2009). Determination of Sinapic Acid Derivatives in Canola Extracts Using High-Performance Liquid Chromatography. *Journal of the American Chemical Society, 87(2)*, 147-155. doi: 10.1007/s11746-009-1486-0.

[32] Gerzhova, A., Monor, M., Benali, M., & Aider, M. (2016). Study of total dry matter and protein extraction from canola meal as affected by the pH, salt edition and use of zeta-potential/turbidimetry analysis to optimize the extraction conditions. *Food Chemistry, 201,* 243–252. doi: 10.1016/j.foodchem.2016.01.074

[33] Daun, J. K., Eskin, N.A., & Hickling, D. (2011). Canola- Chemistry, Production, Processing and Utilization. AOCS Press. Pages 119-162.

[34] Krimpen M., Veldkamp, T. (2017) New alternative protein sources: their potential contribution. Livestock Research, Wageningen University and Research.

[35] Borisjuk, L., Neuberger, T., Schwender, J., Heinzel N., Sunderhaus, S., Fuchs, J., Rolletschek, H. (2013). Seed Architecture Shapes Embryo Metabolism in Oilseed Rape. The Plant Cell, 25(5), 1625-1640. doi: 10.1105/tpc.113.111740

[36] Hu, Z., Hua, W., Zhang, L., Deng, L., Wang, X., Liu, G., Wang, H. (2013). Seed Structure Characteristics to Form Ultrahigh Oil Content in Rapeseed. *PLoS ONE, 8(4).* Doi: 10.1371/journal.pone.0062099

[37] Campbell, T.C. (2015). Animal vs. Plant Protein. Retrieved December 14, 2017, from http://nutritionstudies.org/animal-vs-plant-protein/

[38] Tzen, J., Cao, Y., Laurent, P., Ratnayake C.,& Huang A. (1993). Lipids, Proteins, and Structure of Seed Oil Bodies from Diverse Species. *Plant Physiology, 101*(1), 267-276. doi: 10.1104/pp.101.1.267

[39] Tandang-Silvas, M. R., Fukuda, T., Fukuda, C., Prak, K., Cabanos, C., K,mura, A., Maruyama, N. (2010). Conservation and divergence on plant seed 11S globulins based on crystal structures. Biochimica Et Biophysica Acta (BBA) – Proteins and Proteomics, 1804(7), 1432-1442. Doi: 10.1016/j.bbapap.2010.02.016

[40] Rico, M., Bruix, M., Gonzalez, C., Monsalve, R. I., & Rodriguez, R. (1996) 1H NMR Assignment and Global Fold of Napin BnIB, a Representative 2S Albumin Seed Protein. *Biochemistry, 35*(49), 15672-15682. Doi: 10.1021/bi961748q

[41] Maurer, S., Ghebremedhin, M., Zielbauer, B. I., Knorr, D; Vilgis, T. (2016). Retrieved January 11, 2018, from http://www.mpip-mainz.mpg.de/4120261/Oil_bodies_from_plant_seeds

[42] Shimada, T.L. & Hara-Nishimura, I. (2010). Oil-Body-Membrane Proteins and Their Physiological Functions in Plants. *Biological &. Pharmaceutical Buletin, 33*(3),360—363. doi: 10.1248/bpb.33.360

[43] Geerts M.E.J., Mienis, E., Nikiforidis, C.V., Padt A., & Goot, A.J. (2017). Mildly refined fractions of yellow peas show rich behavior in thickened oil-in-water emulsions. *Innovative Food Science & Emerging Technologies, 41,* 251–258. doi: 10.1016/j.ifset.2017.03.009

[44] Nikiforidis, C.V., Matsakidou, A., & Kiosseoglou, V. (2014). Composition, properties and potential food applications of natural emulsions and cream materials based on oil bodies. *RSC Adv., 4*(48), 25067-25078. doiI: 10.1039/c4ra00903g

[45] Mcclements, D.J. (2007). Critical Review of Techniques and Methodologies for Characterization of Emulsion Stability. *Critical Reviews in Food Science and Nutrition, 47(7),* 611-649. Doi: 10.1080/10408390701289292

[46] Daun, J.K. (1999). The effect of glucosinolates on the nitrogen to protein ratio for rapeseed and canola; Bull. GCIRC 1999, 16, 58-63.

[47] Food and Agriculture Organization of the United Nations (FAO). (1986). 7. Food analysis: general techniques, additives, contaminants and composition. Manuals of Food Quality Control, FAO Food and Nutrition Paper 14/7.

[48] Rosental, A., Pyle, D.L., Niranjan, K. Aqueous and enzymatic process for edible oil extraction*.* (1996). *Department of Food Science and Technology*. *Enzyme and Microbial Technology 19*, 402-420.

[49] Nikiforidis, C. V. &, Kiosseoglou, V. (2009). Aqueous Extraction of Oil bodies from Maize Germ (Zea mays) and Characterization of the Resulting Natural Oil-in Water Emulsion*. Journal of Agricultural and Food Chem*istry, *57*(12), 5591-5596.doi: 10.1021/jf900771v

[50] Rosental, A., Pyle, D. L., & Niranjan, K. (1998). Simultaneous Aqueous Extraction of Oil and Protein from Soybean: Mechanisms for Process Design. *Department of Food and Bioproducts Processing, 76(4),* 224-230. doi: 10.1205/096030898532124

[51] Campbell K. A., & Glatz C.E. (2009). Mechanism of Aqueous Extraction of Soybean Oil. *Journal of Agricultural and Food Chemistry, 57*(22), 10904-10912. doi: 10.1021/j902298a

[52] Wanasundara, J., & McIntosh, T. (2008). U.S. Patent No. US8557963. Washington, DC: U.S. Patent and Trademark Office.

[53] Aluko, R. E., & McIntosh, T. (2001). Polypeptide profile and functional properties of defatted meals and protein isolates of canola seeds. *Journal of Food and Agriculture, 81*(4):391–396. doi: 10.1002/1097-0010(200103)81:43.0co;2-s

[54] Aluko, R.E., McIntosh, T., & Katepa-Mupondwa, F. (2005). Comparative study of the polypeptide profiles and functional properties of *Sinapis alba* and *Brassica juncea* seed meals and protein concentrates. *Journal of the Science Food and Agriculture, 85*(11), 1931–1937. doi: 10.1002/jsfa.2199

[55] Ghodsvali, A., Khodaparast, M. H., Vosoughi, M., Diosady, L. (2005). Preparation of canola protein materials using membrane technology and evaluation of meals functional properties. Food Research International, 38(2), 223–31. Doi: 10.1016/j.foodres.2004.10.007

[56] Food and Agriculture Organization of The United Nations (FAO). (2015). Codex Alimentarius-International Food Standards, Standard for named vegetable oils. CODEX STAN 210-1999.

[57] Food and Agriculture Organization of The United Nations (FAO) Food and Nutrition Paper. (1986). Manuals of food quality control. 7. Food analysis: general techniques, additives, contaminants and composition.

[58] Greenwood, R. (2003). Review of the measurement of zeta potentials in concentrated aqueous suspensions using electroacoustic. *Advances in Colloid and Interface Science, 106* (1-3), 55-81. Doi: 10.1016/s0001-8686(03)00105-2

[59] Fennema, O. R., Damodaran, S., & Parkin, K. L. (2008). *Fennema's Food Chemistry 4th Eddition*. Boca Raton: CRC Press. Page, 784-843.

[60] Cunningham, N. (2013). Examples of Rheology Testing Techniques. Retrieved March 11, 2018, from http://www.rheologyschool.com/testing/testing-examples

[61] Chaplin, M. (2018). Hydrocolloid Rheology. Retrieved March 11, 2018, from http://www1.lsbu.ac.uk/water/rheology.html

[62] Malvern Panalytical. (2018). Rheological Analysis of Dispersions by Frequency Sweep Testing. Retrieved March 11, 2018, from https://www.azom.com/article.aspx?ArticleID=2884

[63] Rustom, I. Y., Lopez-Leiva, M., & Nair, B.M. (1991). A Study of Factors affecting extraction of peanut (Arachis hypogaea L.) solids with water. *Food Chemistry 42*(2), 153-165. doi: 10.1016/0308-8146(91)90031-i

[64] Wu, J. & Muir, A.D. (2008). Comparative Structural, Emulsifying and Biological Properties of 2 Major Canola Proteins, Cruciferin and Napin. *Journal of Food Science*, *73*(3). doi: 10.1111/j.1750-3841.2008.00675.x

[65] Nikiforidis, C., & Kiosseoglou, V. (2010). Physicochemical Stability of Maize Germ Oil Body Emulsions as Influenced by Oil Body Surface-Xanthan Gum Interactions. *Journal of the Agricultural and Food Chemistry*, *58*(1), 527-532. doi: 10.1021/jf902544j

[66] Nikiforidis, C., Kiosseoglou, V., & Scholten, E. (2013). Oil Bodies: An insight on their microstructure-maize germ vs sunflower seed. *Food Research International 52*(1), 136-141.
doi: 10.1016/j.foodres.2013.02.052

[67] Nikiforidis, C.V., Biliaderis, C.G., & Kiosseogluo, V. (2012). Rheological characteristics and physicochemical stability of dressing-type emulsions made of oil bodies-egg yolk blends. *Food Chemistry, 134*(1)*,* 64-73. doi: 10.1016/j.foodchem.2012.02.058

[68] Nikiforidis, C.V., Ampatzidis, C., Lalou, S., Scholten, E., Karapantsios, T.D., & Kiosseoglou, V. (2013). Purified oleosins at air-water interfaces. *Soft Matter*, *9*(4), 1354-1363. doi:10.1039/c2sm27118d

[69] White, D., Fisk, I., Mitchell J., Wolf, B., Hill, S., & Gray D. (2006). Sunflower-seed oil body emulsions: Rheology and stability assessment of a natural emulsion*. Food Hydrocolloids 22*(7), 1224-1232. Doi: 10.1016/j.foodhyd.2007.07.004

[70] Mantovani, R. A., Furtado G. D., Netto, F. M., & Cunha, R., L. (2018). Assessing the potential of whey protein fibril as emulsifier*. Journal of Food Engineering 223,* 99-108. Doi: 10.1016/j.jfoodeng.2017.12.006