

Utilization of Hempseed Press Cake

Optimization of Protein Isolation and Ice Cream Formulation

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Master Thesis Project

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Abstract

Hempseeds, a by-product from hemp fiber production, have a very good nutritional profile, both for essential fatty acids and amino acids, increasing the interest to use hempseeds as a food source. The aims of this project were to optimize the extraction of proteins from hempseed press cake, characterize the functions of the proteins and determine if an ice cream formulation can be created.

The extraction of proteins was based on a method for alkali extraction with isoelectric precipitation. Three different optimizations were investigated, different alkali pH treatments, different extraction times for the alkali extraction and a test to scale up to semi-pilot scale. The results showed that the most optimal treatment was when the alkali pH was kept constant at 10.5 with 1 h extraction time. This yielded in a protein rich sediment with 90% protein content on dry basis and a 60% yield. It was also shown that the process could be scaled up to semi-pilot scale. The characterization was made by examining the proteins emulsifying properties, thermal behavior by differential scanning calorimetry (DSC) and viscosity behavior by a rapid visco analyzer (RVA). The results showed that the proteins have an ability to work as an emulsifier, it also has a thermal transition at approximately 80°C. Whereas the RVA showed no heat induced viscosity change. The ice cream formulations were created with different protein to fat to water (PFW) ratios and compared to an ice cream reference. The ice cream with the properties most similar to the ice cream reference had a PFW ratio of 1:4.5:12 with equal amounts of rapeseed oil and coconut oil as a fat source and fresh sediment as protein source. This concludes that it was possible to create an ice cream formulation based on hempseed proteins.

Populärvetenskaplig Sammanfattning

Efterfrågan av nya växtbaserade proteinkällor växer hela tiden. Fler och fler tittar på växtbaserade proteiner dels ur hälsoaspekter men även för att få en låg klimatpåverkan. Hampa är ett bra alternativ då odling av växten har en väldigt låg klimatpåverkan. Genom att utvinna proteiner ur hampapresskakan, vilken är en biprodukt från fiber- och hampoljetillverkningen, kan en växtbaserad proteinkälla skapas. Dessa proteiner, som har väldigt högt näringssvärde, kan sedan användas i olika livsmedelsformuleringar.

Cannabis Sativa L. även kallad hampa har historiskt sett använts för dess starka fiber, livsmedel och medicin. Idag är viss hampodling olaglig på grund av dess innehåll av aktiva substanser så som δ-9-tetrahydrocannabinol (THC). Det har dock tagits fram en variant, även kallad industrihampa, som inte innehåller THC. Denna variant används för bland annat fiberproduktion, vilket ger en biprodukt i form av hampafrön som i sin tur kan användas för att producera hampolja. Utvinningen av hampolja skapar ännu en biprodukt, nämligen hampapresskaka, vilket är det som blir kvar efter pressning. Presskakan innehåller ungefär 25% protein av väldigt hög kvalitet.

Det här projektet har fokuserat på hur man kan utvinna proteiner från hampafrökaka för att få ut så mycket protein som möjligt med en hög renhet. Utöver utvinning av proteiner, har även proteinernas egenskaper undersökts och till sist har en formulering för glass baserat på hampaprotein tagits fram. Vid utvinningen av proteiner kunde ungefär 60% utav proteinerna utvinnas ur frökakan. Proteinerna som utvanns hade en hög renhet på cirka 90% protein på torr bas. Med torr bas menas det som finns kvar om man tar bort allt vatten. Av de utvunna proteinerna kunde en glass formuleras som har en konsistens som liknar en gräddbaserad glass.

Tre olika parametrar för utvinningen av proteiner har undersökts, vilket pH som används vid lösning av proteinerna, hur lång tid lösningen ska behandlas med det specifika pH och om man kan skala upp produktionen. Utvinningen av proteiner går till som så att presskakan mals och blandas med vatten. Sedan höjs pH på lösningen för att proteinerna ska laddas och kunna vara lösta i vattenfasen. Den här processen pågår under en viss tid. Detta skapar två strömmar, en ström med allt som inte gick att lösa i vattenfasen och en ström med det som gick att lösa i vattenfasen. De lösta proteinerna sparar och pH sänks igen för att laddningarna ska neutraliseras. Detta gör att proteinerna inte kan vara lösta i vattnet längre utan kommer att klumpas ihop och sjunka till botten. Detta skapar två nya strömmar, strömmen med vattnet och den nya strömmen med alla ihop klumpade proteiner.

Resultaten från det här projektet visar att det är möjligt att utvinna protein från hampafrökaka även i lite större skala vilket är viktigt för en framtida industriell utvinning. Det visar även att det är möjligt att formulera livsmedelsprodukter, som till exempel glass av proteinerna. Baserat på resultaten kan fler undersökningar göras för att formulera andra typer av livsmedel.

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1 Abbreviations

Different abbreviations have been used throughout the report. A summary can be found in Table 1.

Table 1 Abbreviations used in the report.

THC	δ-9-tetrahydrocannabinol
d ₃₂	Surface weighted mean
d ₄₃	Volume weighted mean
DSC	Differential Scanning Calorimetry
RVA	Rapid Visco Analyzer
ΔH	Transition enthalpy
T _o	Onset temperature
T _p	Peak temperature
T _c	Conclusion temperature
DM	Dry matter content
P _{Sediment}	Protein concentration in sediment
P _{DM}	Protein concentration in dried sediment
P _{Press cake}	Protein concentration in press cake
P _{Solid phase}	Protein concentration in solid phase
EC	Extraction coefficient
PC	Precipitation coefficient
UGLM	Univariate General Linear Model
ANOVA	Analysis of variance
PFW ratio	Protein to fat to water ratio

2 Background

2.1 Introduction

Hempseeds and hempseed press cake are by-products from the production and processing of hemp fibers and hemp oil. Today the hempseed press cake is used as animal feed instead of using it for human consumption (Gunnarshög, 2020). With the good nutritional value of both hemp oil and proteins from hempseeds, the interest in characterizing the proteins and introduce it to human consumption is increasing (Tang et al., 2006). And since the demand for plant-based protein sources are increasing, hempseed proteins are a good candidate in producing a new plant-based protein source that can be used for further food formulations. This project will be performed in collaboration between Lund University, Mossagården and BoFood and will examine the possibilities to use hempseed press cake to extract proteins for formulation of foodstuff. Since BoFood is a plant-based dairy company and Mossagården grows organic hemp, a suiting food product to examine is a hemp-based ice cream.

2.2 Aim

The aim of this master thesis project was divided into three parts.

- To optimize the protein extraction from hempseed press cake, a by-product from hemp oil production.
- To characterize the functional properties of the extracted hempseed protein. Functional properties related to ice cream formulation will be investigated.
- Determine if an ice-cream formulation can be created from the extracted proteins.

2.3 *Cannabis Sativa L.*

Cannabis Sativa L., also called industrial hemp, is an annual herbaceous plant that has been widely used throughout history for its strong fibers, food, medicine and drugs. For instance, hemp has been used as a source of food and medicine in China for over 3000 years (Callaway, 2004). Due to hemp containing different active substances, like δ -9-tetrahydrocannabinol (THC), which is used as a drug component, its use has been prohibited in many countries. There is, however, varieties of hemp that has been legalized as they contain less than 0.3% of THC. These cultivars are referred to as industrial hemp, and its legislation has greatly increased the interest of hemp-based products (Tang et al., 2006).

Industrial hemp is a very fast-growing crop that matures in approximately four months and requires very low maintenance. Hemp can be cultivated in many different climates and soils, and even grow in high latitudes, like Sweden. It is also a good crop for organic cultivation and organic crop rotation. It can grow well without the use of pesticides, herbicides and fungicides and its fast growth with a long root system is efficient for not only suppressing weeds, but it also increases soil health. When harvesting the plants, 40% of the hemp biomass is left on the field as leaves and roots, working to fertilize the soil further by returning nutrients. It is also a plant that is five times more efficient at binding carbon dioxide than a forest with the same size, making it a good option in decreasing greenhouse gases (Aluko R. E., 2017; Callaway J. C., 2004a; Callaway J. C., 2004b).

2.4 Hempseeds

Hempseeds are an oilseed that contain approximately 30% oil and 25% protein together with some insoluble fibers. Hempseed oil is regarded as an oil with high nutritional value since it consists of over 80% polyunsaturated fatty acids with very good essential fatty acid composition of both linoleic acid (omega-6) and α-linolenic acid (omega-3). The proteins are also regarded to have high nutritional value since it has an amino acid composition with a sufficient amount of essential amino acids. (Tang et al., 2006; Aluko R. E., 2017).

The seeds contain two major storage proteins, globulin and albumin. The globulin is a protein called Edestin and it contributes to approximately 75% of the total protein content. It consists of two subunits linked by a sulfide bond and it has a molecular weight of approximately 300 kDa. Albumin is the second most abundant protein contributing to approximately 25% of the total protein content. The albumin has less sulfide bonds than Edestin, making it more flexible in its structure. A small (10 kDa) methionine- and cysteine-rich protein has also been isolated from hempseeds (Aluko R. E., 2017; Tang et al., 2006).

Something to also have in mind is the presence of antinutrients that are common in plant seeds. Antinutrients, are substances, that interfere with the absorption of nutrients and thus lowering the bioavailability. Phytic acid is a common form of stored phosphorous in plant seeds. When being consumed, it works as an antinutrient that decreases the bioavailability of proteins and minerals. It decreases the digestibility of proteins and forms complexes with minerals like zinc. Phytic acid together with other antinutritional compounds have been found in hempseeds, even though the amount varies with different hemp varieties (Russo and Reggiani, 2013; Russo and Reggiani, 2015).

2.5 Existing Hemp Based Products on the Market

There is an increasing interest in hempseed products due to the functionality and nutritional value of hemp proteins. Several different commercial products are on the market, mainly products derived from the press cake, to function as a source of plant protein and fiber (House et al., 2010). These products include hemp oil, hemp milk, protein powders and hemp seeds on the market today (GoodHemp, 2020). There even seem to be a version of a non-dairy hemp-based ice cream on the Canadian market (Coolhemp, 2020).

2.6 Cold-Pressed Hempseed Press Cake

The hempseed press cake is a by-product from oil manufacturing and the press cake used was produced by cold-pressing. Before pressing, the seeds are rinsed and dried. The seeds were mechanically pressed to retrieve the oil, without the temperature exceeding 35°C. Not all oil is pressed from the seeds but remain in the press cake. The oil is being filtered to separate it from shell residues that are later added to the press cake. In this way, no waste is produced since the press cake is used as animal feed (Gunnarshög, 2020).

2.7 Extraction of Hempseed Proteins from Press Cake

The method used was based on a common method of extracting hempseed protein by alkali extraction with isoelectric precipitation (Wang et al., 2019). The same type of process is also common for extraction of rapeseed proteins (Von Der Haar, 2014; Rodrigues et al., 2012).

Initially, the press cake was ground into a powder which were dispersed in water. Thereafter, the pH was increased to solubilize the proteins in order to make an alkaline extraction. This part was constructed to soften and disrupt the cell walls in order to free and solubilize the proteins (Malomo et al., 2014; Tang et al., 2006).

After the solubilization of the proteins, the pH is decreased in order to precipitate the proteins by isoelectric precipitation (Malomo et al., 2014; Tang et al., 2006). This means that the pH was decreased to the proteins isoelectric point. This is where the proteins have a net charge of 0 and if the pH is decreased or increased beyond the isoelectric point the proteins will have either an uptake or release of hydrogen ions and get a positive or negative net charge form. When at the isoelectric point, the proteins will coagulate, and an isoelectric precipitation is made (Rodrigues et al., 2012).

2.8 Functional Characterization

2.8.1 Emulsifying Properties

Emulsions are defined as two liquids, where one liquid is dispersed as small droplet in the other liquid. The most common type of emulsions in the food industry are emulsions formulated with oil and water. There are several classifications of emulsion based on the liquids it constitutes of. In this case, the relevant emulsion is oil-in-water emulsions, where oil is dispersed as droplets in water (McClements, 2007).

When an emulsion is formulated, there are several destabilization mechanisms taking place. Creaming and sedimentation are processes that are driven by density differences where the two liquid separates. Flocculation is when droplets aggregate. Coalescence is when smaller droplets merge to form larger droplets (McClements, 2007).

In order to prevent the destabilizing mechanisms different stabilizers can be used. Stabilizers are classified according to how they work. An emulsifier is defined as a substance that can attach to the surface of the emulsion droplets and prevent them from aggregating and merging. The emulsifiers are surface active and can adsorb to the surface of the droplet. One example of emulsifiers are certain proteins (McClements, 2007).

Measuring different characteristics of an emulsion can be done in different ways. One way is to measure the size of the droplets to evaluate how good an emulsifier is of stabilizing the emulsion. An effective emulsifier stabilizes smaller emulsion droplets compared to a less effective emulsifier. Since most emulsions are polydisperse, meaning not all droplets have the same size, gives multiple ways of measuring droplet size. By measuring the mode, is simply put to take the height of the peak of the size distribution curve, the size of which most droplets have. Another way of measuring the size is by different means (McClements, 2007). Two of the most common means are volume weighted mean and surface weighted mean. What kind of mean that is used, depends on what kind of particle distribution the calculations are based on. If the distribution is based on a volume distribution, using the volume of different particles, a volume weighted mean (d_{43}) is used. If the particle distribution is based on particle surface areas, a surface weighted mean (d_{32}) is used. The numbers in d_{32} and d_{43} indicates what kind of distribution was used (Horiba, 2020).

2.8.2 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) is a method of calorimetry that is used to measure how thermal properties change in a material over time and temperature. The measurements are made by heating two pans equally, one containing the sample and one reference that is empty. Since the sample will have a different heat capacity than the reference, there will be a difference in temperature between the sample and the reference and the heat flux can be calculated. Due to thermal changes of the sample, there will be either an uptake or release of heat, based on what kind of change the sample will have. DSC is often used to measure how thermally stable proteins are, and if they will have thermal transition, like denaturation, when being heated. It is a way of characterizing the proteins behavior when adding heat (Gill et al., 2010).

2.8.3 Rapid Visco Analyzer

A Rapid Visco Analyzer (RVA) is a technique to measure rheological properties of a sample. It is often used to characterize the rheological behavior of starch. However, it can also be used for other materials, for instance whey protein concentrates. It measures the gelling abilities of proteins by heating, cooling and applying shear and measuring how the viscosity changes. Measuring the viscoelastic properties of a sample, is a way of characterizing the sample. It is interesting to have knowledge of how the sample behaves during temperature change and different shears, but also to examine the gelling ability in order to predict if a network can be formed. It is a good characterization for future food processing of the material (Onwulata et al., 2013).

2.9 Ice Cream Formulation

Ice cream can be defined as an emulsion that has been frozen while incorporating air into it. The structure of the ice cream is complex, involving several components creating stable emulsions together with fat crystals, air bubbles and other substances as micelles, and ice crystals. The air bubbles and ice crystals that are incorporated into the mix is approximately 20 μm to 40 μm in diameter (Figure 1).

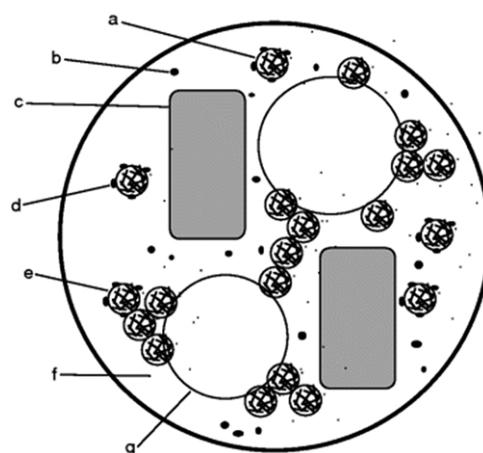


Figure 1 A schematic diagram of structure of ice cream, showing the principal structural elements of fat globules and clusters, air bubbles and ice crystals, all embedded within the free-concentrated unfrozen phase a) Fat globules, partially crystalline; b) Casein micelles; c) Ice crystals; d) Mixed membrane around fat globules of caseins micelles, non-micellar casein, whey proteins, and emulsifier; e) Partially-coalesced fat globule network; f) Freeze-concentrated unfrozen phase of sugars, salts, and un-adsorbed proteins; g) Air bubbles, surrounded by protein membrane and adsorbed fat globules. The figure was retrieved from Goff, H. D. (2015).

The complex structure is usually composed of seven different categories of ingredients. Fat, milk solids (not fat) or plant proteins, sweeteners, emulsifiers, stabilizers, water and flavors. Proteins, in this case can work in more than one of the ingredient categories, both as proteins and as emulsifiers for instance. They have an important role of stabilizing the emulsion and air bubbles as well as holding water. When creating the ice cream, several processing steps are taken. The ice cream mix is prepared and pasteurized, to reduce the risk of pathogens. It is then homogenized, which work as the main emulsion creating step. The new emulsion, is cooled and ripened, allowing the mixture to set and rearrange structure to become creamier. The last step is freezing while being stirred to incorporate air (Goff, H. D., 2015).

3 Materials and Methods

3.1 Materials and Chemicals

After harvest, the hempseeds were cleaned, dried and cold pressed to get out the hempseed oil. A press cake residual is then received which is used in this project. The hempseed press cake was provided by Mossagården, Veberöd. Press cake from hempseeds that was harvested in two different years were used, 2016 and 2018, and both press cakes were stored in freezer at -18°C until the experiments.

The hemp protein powders used in the RVA measurements and the dehulled hempseeds used in the ice cream formulations, was purchased online from Rawfoodshop.se. The hemp oil used in the ice cream formulations was provided by Mossagården, Veberöd. All the other ingredients for the ice cream formulation was purchased at a local supermarket.

All chemicals that were used can be seen in Table 2.

*Table 2 Chemicals used in the experiments. *Chemicals used in mixing the phosphate buffer (0.005 M, 0.2 M NaCl, pH 7)*

Chemical	Producer
Citric acid	VWR International, Radnor, PA, USA
Sodium hydroxide, 2 M	Merck, Darmstadt, Germany
Buffer solution pH 4	VWR International, Radnor, PA, USA
Buffer solution pH 7	VWR International, Radnor, PA, USA
Aspartic acid	Thermo Electron, Milan, Italy
*Sodium hydroxide, 0.1 M	Merck, Darmstadt, Germany
*Di-sodium hydrogen phosphate	Merck, Darmstadt, Germany
*Sodium dihydrogen phosphate	Merck, Darmstadt, Germany
*Sodium chloride	Merck, Darmstadt, Germany
Miglyol 812	Caelo, Hilden, Germany

3.2 Flowchart

The experiments to optimize the extraction of hempseed proteins all followed the general flowchart (Figure 2) with different parameter changes.

The first step in the process was milling, where the press cake was milled into a powder. Thereafter, the extraction was performed, in which the press cake was dispersed into water, and sodium hydroxide was added to increase the pH in the slurry. During the extraction step, the pH was either adjusted in the beginning of the extraction time or held constant during the complete extraction time. The slurry produced from the extraction step was separated into two phases, solid phase and liquid phase. The solid phase contained all the water insoluble components from the press cake, while the liquid phase contained all the water-soluble ones, including the solubilized proteins. Due to the solid phase being heavier than liquid phase they could be separated in separation step 1. The liquid phase was collected, and citric acid was added to decrease the pH and precipitate the proteins in the precipitation step. The precipitation step gave two phases, the supernatant and the sediment. The sediment contained precipitated components, like proteins, and the supernatant contained soluble mater. Since the sediment was

heavier than the supernatant, they could thereby be separated in separation step 2. The sediment, containing a high concentration of proteins was collected as the end-product.

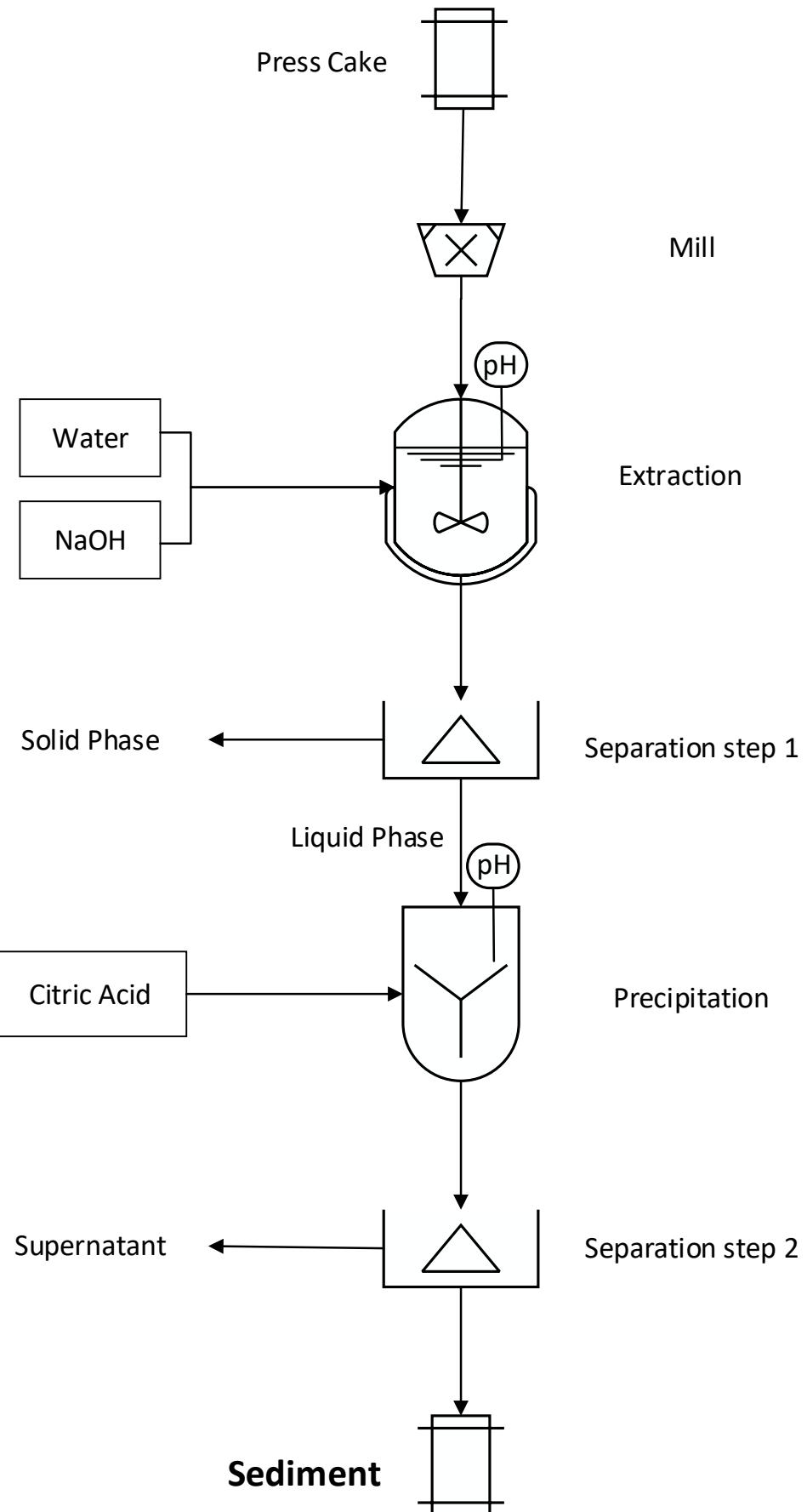


Figure 2 Flowchart of the general process of hempseed protein extraction

3.3 Optimization of the Extraction of Hempseed Proteins

In order to optimize the extraction of hempseed proteins to increase the overall protein yield, different parameters were examined. The optimal pH for the extraction step as well as whether the pH should be held constant or not. Also, the extraction time of the extraction step and in the end, a scale up to semi-pilot was performed to produce enough material for the ice-cream part.

3.3.1 Different pH Treatments

Six different pH treatments were tested in this optimization. Three treatments were done with a constant pH and three pH were done with a non-constant pH. An overview of the six treatments can be found in Figure 3.

3.3.1.1 Non-Constant pH in the Extraction Step

The following parameters were applied to the general process (Figure 2). 50 g of hempseed press cake from 2016 was weighed (Sartorius Entris Laboratory balance) and grounded using a knife mill (Retsch Grindomix GM200) at 2500 rpm for 20 seconds in 4 second intervals. The press cake powder was thereafter placed in a 1000 ml plastic container and 450 g of water was added, giving a press cake to water ratio of 1:9.

In the extraction step, three different pH were tested, pH 10, 10.5 and 11. The pH in the slurry was adjusted to the examined pH with 2 M NaOH. After 10 min of stirring at 750 rpm with a Rushton impeller (IKA Microstar 7.5 control) the pH was re-adjusted to the examined pH. Counting from the first pH adjustment, the slurry was stirred at 750 rpm for 4 h. The experiment was performed in triplicates.

After the extraction, the pH was noted, and the slurry was transferred into a centrifugal tube (750 ml). The slurry was centrifuged (Beckman Coulter Avanti J-15R Centrifuge) at 4700 rpm for 20 min at 20°C. The centrifugal step separated the slurry into two phases, a liquid phase and a solid phase. The liquid phase was collected for the next step of the recovery process while the solid phase was saved for further analyses.

The experiment continued with the precipitation step for the liquid phase. Citric acid was added to lower the pH to 5.5. After the precipitation, the slurry was centrifuged (Beckman Coulter Avanti J-15R Centrifuge) at 4700 rpm for 20 min at 20°C. The supernatant and sediment were separated and collected for further analysis. All samples were stored in plastic containers at -18°C.

3.3.1.2 Constant pH in the Extraction Step

The following parameters were applied to the general process (Figure 2). The method used was the same as for Non-Constant pH extraction (3.3.1.1). But instead of just readjusting the pH during the extraction step, the pH was held constant during the whole extraction. Three different pH were tested, pH 10, 10.5 and 11. The pH in the slurry was adjusted to the examined pH with 2 M NaOH and kept constant with a small bioreactor control (Belach Bioteknik AB, Model CP10/ SARA) during the 4 h extraction time in the extraction step. The slurry was stirred at 750 rpm with a Rushton impeller (IKA Microstar 7.5 control) the entire extraction time.

3.3.2 Different Extraction Times

Four different extraction times were tested using one pH treatment. The pH treatments chosen was constant pH 10.5 (Figure 3).

The following parameters were applied to the general process (Figure 2). The method used was the same as for constant pH extraction (3.3.1.2). The pH in the slurry was adjusted to pH 10.5 with 2 M NaOH and kept constant with a small bioreactor control (Belach Bioteknik AB, Model CP10/ SARA). The parameter changed was the extraction time. 1 h, 2 h, 3 h and 4h were tested and evaluated. Three replicates were made for 1 h and 4 h and a screening with one replicate was made for 2 h and 3 h.

3.3.3 Scaling Up to Semi-Pilot Scale

A test was done in a larger scale. Only one test was done with the chosen pH treatment of Constant pH 10.5 and an extraction time of 4 h (Figure 3).

The following parameters were applied to the general process (Figure 2). A test was performed once in semi-pilot scale. 2 kg of hempseed press cake from 2018 was weighed (Sartorius Entris Laboratory balance) in 500 g portions and milled with a knife mill (Robot Coupe R302 v.v.) at 3000 rpm for 3 min. The press cake powder was thereafter placed in a stirred tank and 18 liters of water was added, giving a press cake to water ratio of 1:9.

The pH of the slurry was adjusted to 10.5 with 2 M NaOH. Counting from the first pH adjustment, the slurry had an extraction time of 4 h with continuous stirring at 205 rpm (IKA RW 28 digital). The pH was kept constant by manually adding 2 M NaOH. During the first hour of extraction, the pH was adjusted to 10.5 when it reached pH 10.4. After 1 h, the pH was adjusted to pH 10.5 every 10 min.

The first separation step was performed by a decanter (Lemitech MD80, Germany, 2018). The separation was made with 6687 rates of evolution (acceleration of 2000 g) and a differential force of 10 rpm. The weir disc size used was 56 mm. The capacity into the decanter was set to approximately 20 l/h with a peristaltic pump (Masterflex Easyload Model 77200-62, Cole-Parmer, USA). The decanter separated the slurry into two different streams: a solid phase and a liquid phase. Both phases were collected, and the liquid phase was used in the next process step.

The liquid phase was distributed into centrifugal tubes (750 ml) and citric acid was added to lower the pH to 5.5. After the precipitation, the tubes with the slurry was centrifuged (Beckman Coulter Avanti J-15R Centrifuge) at 4700 rpm for 20 min at 20°C. The supernatant was sampled, and the sediment were collected for further analysis.

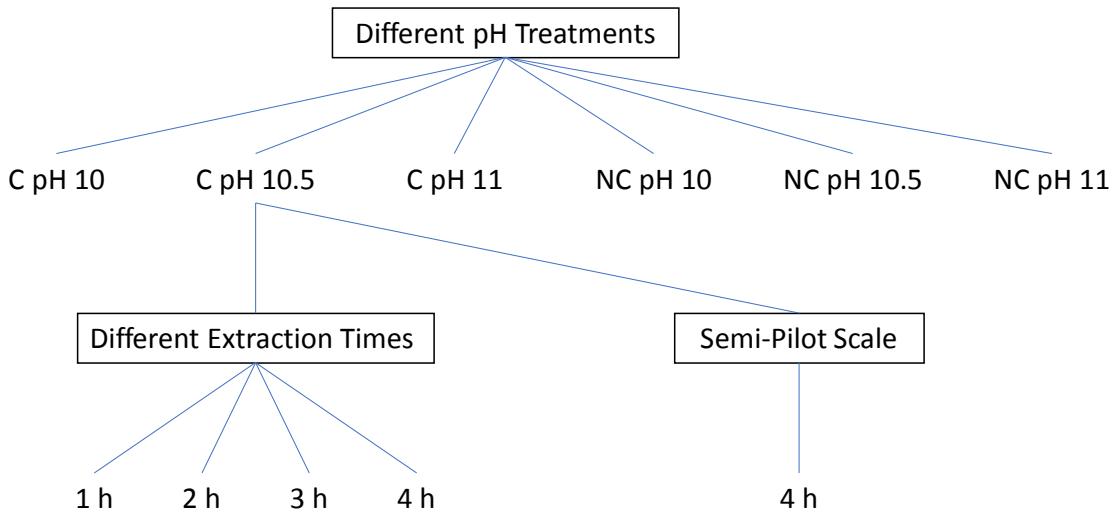


Figure 3 An overview of the different optimizations that were done and what pH treatment and extraction time that were used in the different experiments. C stands for Constant and NC stands for Non-Constant.

3.4 Analysis

3.4.1 Dry Matter Content

To determine the dry matter content of the solid phase and the sediment, both oven drying and freeze-drying methods were used.

3.4.1.1 *Oven Drying*

Oven drying were used for determining the dry matter content of the solid phase and the sediment from all extraction experiments (3.3). The analysis was performed in triplicates on the sediment and duplicates on the solid phase. 3-5 g of sample was weighed in a metal cup and placed in a convection oven (Fermaks). The samples were dried at 103°C, for at least 16 h, until constant weight was reached. After drying, the samples were placed in a desiccator for at least 20 min to ensure constant weight and re-weighed.

3.4.1.2 *Freeze Drying*

Freeze drying was used to produce material for further analysis on the sediment. Approximately 45 g of sample was weighed in aluminum boxes and placed in a freeze dryer (Hetrosicc, Heto Lab Equipment, Birkerød, Denmark) and let dry for 5 days. After the drying, the samples were placed in a desiccator for at least 24 h to ensure constant weight and re-weighed.

3.4.2 Protein Analysis

The hempseed press cake, the solid phase and the sediment from all extraction experiments (3.3) were analyzed for protein content (Thermo Electron Corporation, Flash EA 1112 Series N/Protein Analyzer). The oven dried sediment was used for the measurements of the sediment, otherwise the original samples from the press cake and the solid phase was used. Between 25 and 50 mg of sample was weighed (Entris Mettler AE 163) in an aluminum cup. The nitrogen content was measured, and the protein content was calculated with a protein conversion factor of 6.25. The solid phase was analyzed in duplicates and the sediment was analyzed in triplicates.

3.4.3 Emulsifying Properties

The method used was the same as for Östbring et al. (2015). Oil-in-water emulsions were produced in duplicates by mixing 2 ml phosphate buffer (0.005 M, 0.2 M NaCl, pH 7), with 1 ml Miglyol 812 and varying amounts of hemp protein-rich sediment in glass tubes. The sediment was added to formulate emulsions with 2, 4, 8, 16 and 32 mg of hempseed protein/ml oil. The emulsions were mixed (Ystral D-79282, Ballrechten-Dottingen, Germany) for 1 min at 22 000 rpm and thereafter incubated at 4°C for 1 h. After the extraction, the particle size was measured by light scattering (Malvern Mastersizer 2000 Ver 5.60, Worcestershire, UK). The pump that transfers the emulsion from the dispersing unit to the measurement chamber was set to 2000 rpm. The emulsions were inverted three times to allow representative sampling and sample was added dropwise to MilliQ water in the dispersion unit to get an obscuration rate of 10-20%. The refractive index (RI) used was 1.45 for the Miglyol oil and 1.33 for water. Each emulsion replicate was measured two times. From the measurements, three different means were calculated: surface weighted mean (d_{32}), volume weighted mean (d_{43}) and mode.

3.4.4 Differential Scanning Calorimeter

To evaluate how the sediment behaved during heating, and if there would be any phase transitions a Seiko 6200 differential scanning calorimeter (Seiko Instruments Inc., Shizuoka, Japan) was used. 2 mg of freeze-dried sample were weighed into aluminum pans and 6 µl of double distilled water was added. The pans were hermetically sealed and heated from 20°C to 160°C at a rate of 10°C per min. An empty pan was used as a reference. The dry matter content for the pans were measured by drying the punctured pans at 105°C for 24 h before re-weighing. The data was analyzed using EXSTAR 6000 thermal analysis system (Seiko Instruments Inc., Shizuoka, Japan). The parameters analyzed was the transition enthalpies (ΔH), where the transition started, called the onset temperature (T_o), the top of the peak, called the peak temperature (T_p) and where the transition ended, called the conclusion temperature (T_c).

3.4.5 Rapid Visco Analyzer

The gelling properties were analyzed using the rapid visco analyzer RVA4500 (Perten Instruments). Both freeze-dried sediment and bought hemp protein powder with 35%, 50% and 70% were analyzed. 4.50 g of sample was used for the hemp protein powder and 3.50 and 4.50 g sample was used for freeze-dried sediment. The water added to the protein powder sample was 26.75 g for 70%, 24.66 g for 50% and 18.20 g for 35%. The water added to the freeze dried sediment was 28.69 g to the 4.50 g sample and 28.56 g water to the 3.50 g sample. The moisture contents used was 1% for the freeze-dried sediment, 6.8% for protein powder 70%, 13% for protein powder 50% and 32.5% for protein powder 35%. The temperature profile (Table 3) was the same as used by (Onwulata et.al., 2013).

Table 3 The temperature profile used for the RVA measurements.

Time	Type	Value
00:00:00	Temperature	50°C
00:00:00	Speed	960 rpm
00:00:10	Speed	160 rpm
00:01:00	Temperature	50°C
00:04:45	Temperature	95°C
00:07:25	Temperature	95°C
00:11:10	Temperature	50°C
00:13:10	End	

3.5 Calculations

The dry matter content was calculated using Equation 1, where DM is the dry matter content.

$$DM (\%) = \left(1 - \frac{Weight_{Before\ drying} - Weight_{After\ drying}}{Weight_{Before\ drying}} \right) * 100 \quad \text{Eq. (1)}$$

To calculate the protein content in the sediment (Equation 2), the result from the protein analysis was used. P_{DM} is the concentration in the dried sediment and P_{Sediment} is the protein concentration in the sediment before drying.

$$P_{Sediment} (\%) = \left(\frac{P_{DM} * Weight_{Before\ drying}}{Weight_{After\ drying}} \right) * 100 \quad \text{Eq (2)}$$

The yield was based on the protein content in the hempseed press cake in comparison to the protein content of the extracted sediment and was calculated according to Equation 3, where P_{Press cake} is the protein concentration in the press cake.

$$Yield (\%) = \left(\frac{P_{Sediment} * Weight_{Sediment}}{P_{Press\ cake} * Weight_{Press\ cake}} \right) * 100 \quad \text{Eq (3)}$$

The extraction coefficient is a measurement of how much protein extracted from the press cake that was located in the liquid phase compared to the solid phase. The extraction coefficient (EC_{Liquid phase}) is equaled to the protein concentration in the liquid phase and was calculated according to Equation 4 and the extraction coefficient (EC_{Solid phase}) calculated according to Equation 5. P_{Press cake} is the protein concentration in the press cake and P_{Solid phase} is the protein concentration in the solid phase.

$$EC_{Liquid\ phase} (\%) = \left(\frac{P_{Press\ cake} * Weight_{Press\ cake} - P_{Solid\ phase} * Weight_{Solid\ phase}}{P_{Press\ cake} * Weight_{Press\ cake}} \right) * 100 \quad \text{Eq (4)}$$

$$EC_{Solid\ phase} (\%) = \left(\frac{P_{Solid\ phase} * Weight_{Solid\ phase}}{P_{Press\ cake} * Weight_{Press\ cake}} \right) * 100 \quad \text{Eq (5)}$$

Lastly, the precipitation coefficient (PC_{Sediment}) is equaled to the protein concentration in the sediment and the precipitation coefficient (PC_{Supernatant}) is equaled to the protein concentration in the supernatant. It is a measurement of how much protein that was precipitated to the sediment in comparison to what was left in the supernatant. This was calculated using Equations

6 and 7, where P_{Sediment} is the protein concentration in the sediment, $P_{\text{Press cake}}$ is the protein concentration in the press cake and $P_{\text{Solid phase}}$ is the protein concentration in the solid phase.

$$PC_{\text{Sediment}}(\%) = \left(\frac{P_{\text{Sediment}} * Weight_{\text{Sediment}}}{P_{\text{Press cake}} * Weight_{\text{Press cake}} - P_{\text{Solid phase}} * Weight_{\text{Solid phase}}} \right) * 100 \quad \text{Eq (6)}$$

$$PC_{\text{Supernatant}}(\%) = \left(\frac{P_{\text{Press cake}} * Weight_{\text{Press cake}} - P_{\text{Solid phase}} * Weight_{\text{Solid phase}} - P_{\text{Sediment}} * Weight_{\text{Sediment}}}{P_{\text{Press cake}} * Weight_{\text{Press cake}} - P_{\text{Solid phase}} * Weight_{\text{Solid phase}}} \right) * 100 \quad \text{Eq (7)}$$

3.6 Statistical Analysis

At least triplicates were made in order to calculate mean values with standard deviations, when possible. A Univariate General Linear Model with a Tukey Post-Hoc Test (UGLM) using SPSS Statistics for Windows, version 24.0 (IBM Corp., Armonk, N.Y., USA) was used to search for outliers and calculate the significance for dry matter content, yield, extraction coefficient and precipitation coefficient for the pH treatments. Significant difference were defined by $p < 0.05$. For the protein concentrations, t-test: paired samples assuming equal variances using Microsoft Excel for Office (Microsoft Inc.), were used and significant difference were defined by $p < 0.05$. The statistical analysis, including an outlier test, for different extraction times were made between 1 h and 4 h using One-Way ANOVA in SPSS Statistics for Windows, version 24.0 (IBM Corp., Armonk, N.Y., USA). When t-test were made, outliers tests were performed with GraphPad outlier calculator (2018 GraphPad Software).

3.7 Ice Cream Formulation

From the extracted hempseed protein rich sediment, different formulations were tried in order to create a hemp protein-based ice cream with a texture, look and mouthfeel that can be compared to a cream-based ice cream. A hemp heart ice cream was also made from dehulled hempseeds. All ice creams were made with an Musso L2 Stella ice cream machine (Musso S.r.l, Italy).

3.7.1 Ice Cream Reference

Ice cream 1 is a cream-based ice cream that was made as a reference. 250 g cream with 38% fat, 250 g milk with 3 % fat and the content of 1 vanilla rod was heated until the mixture was just boiling. The cream-milk was let to cool for 5 min. 70 g of egg yolk and 100 g sugar were beaten until fluffy and carefully added to the creamy milk while vigorously stirring. The batter was heated to 84°C, then cooled to lukewarm in a water bath. The mixture was frozen in an ice cream machine until solid and stored in freezer at -18°C.

3.7.2 Hemp Heart Ice Cream

Ice cream 2 was an ice cream based on dehulled hempseeds and were made with vanilla flavor. Firstly, a hemp milk was made by mixing 40 g dehulled hempseeds with 400 ml water in a blender (Bosch VitaBoost MMBH6P6B, Munich, Germany). The ice cream was made by mixing 100 g hempseeds and 100 ml water in a blender (Bosch VitaBoost MMBH6P6B, Munich, Germany). 100 ml hemp milk, 25 g sugar were added, and the mixture was heated until the sugar dissolved. As flavor, 1 rod of vanilla was added during the heating. The batter was then frozen in an ice cream machine until solid and stored in the freezer at -18°C.

Ice cream 3 was made in the same way as ice cream 2, but instead of vanilla, 3 tablespoons of cacao was added together with 2 pinches of salt and 2 teaspoons of vanilla sugar.

3.7.3 Calculations of Protein, Fat and Water Content for Sediment-based Ice Cream

To be able to formulate an ice cream based on the extracted sediment, the recipe for the reference ice cream was recalculated to amount protein, fat and water to have as a starting point. In order to do this, the assumption that milk, cream and egg yolk only constituted of protein, fat and water was made. From the percentage of protein, fat and water in the ingredients, the amount in g was calculated (Table 4). It was also assumed that everything that was not protein or fat, was water.

Table 4 The calculated composition of protein, fat and water in cream, milk and egg yolk from the ice cream reference that were used as a base for the sediment ice creams.

Ingredient	Amount used in recipe (g)	Protein content (%)	Protein (g)	Fat (%)	Fat (g)	Water (g)
Cream	250	2	5	36	90	155
Milk	250	3.5	8.8	3	7.5	233.8
Egg yolk	70	16	11.2	30	21	32.2
Total amount			25		118.5	426.6

From the calculations (Table 4), 25 g protein, 118.5 g fat and 426.6 g were calculated as a base recipe. This gave a protein to fat to water (PFW) ratio of 1 to 4.7 to 17.1. Since sediment was scarce, the batch was reduced, and 12.5 g protein were used with the same approximate PFW ratio for the first sediment-based ice cream. From the protein analysis, the protein content in the sediment was known to 24% protein. Meaning, 12.5 g protein was representative of 52 g sediment.

3.7.4 Sediment-based Ice Cream

The sediment-based ice creams were made from sediment, a fat source of hemp oil, coconut oil, rapeseed oil or a combination of the fats, water and sugar (Table 5). Either pre-produced sediment that had been frozen or fresh sediment were used.

Table 5 The ingredient list for all sediment-based ice cream formulations. The sediment is assumed to constitute of 24% protein and 76% water. The total water is water from the sediment and added water.

Ice cream	Sediment (g)	Protein from sediment (g)	Total fat (g)	Hemp oil	Coconut oil	Rapeseed oil	Total Water (g)	Sugar (g)
4	52	12.5	59	59			233.5	40
5	52	12.5	59		59		233.5	40
6	40	9.6	40		40		110.4	30
7	40	9.6	40		40		110.4	30
8	55	13.2	55		27.5	27.5	151.8	45
9	55	13.2	55		18	37	151.8	45

Ice cream 4 was an ice cream based on sediment produced in semi-pilot scale (3.3.3). The sediment had been stored in the freezer at -18°C and thawed prior to the ice cream formulation. The ice cream was made with an approximate PFW ratio of 1:4.5:19, and the fat source used was hemp oil. Sediment and water were weighed and added to a saucepan. The mixture was mixed by hand whisking and heated to 80°C in order to disperse the proteins into the water phase. The sugar was added and allowed to dissolve. The mixture was then cooled in a water bath until it reached 30°C. The fat was added slowly while whisking vigorously with a hand whisk and the mixture was frozen in an ice cream machine for 5 min until it became solid. The ice cream was stored in a freezer at -18°C.

The method used to create ice cream 5 was the same as for ice cream 4. Ice cream 5 was also based on sediment produced in semi-pilot scale (3.3.3) treated the same way. The approximate PFW ratio is 1:4.5:19 but the fat source used was coconut oil.

Ice cream 6 was made from fresh sediment, produced the same day as the ice cream formulation according to the constant pH extraction at pH 10.5 with 1 h extraction time (3.3.2). The water content was reduced to have an approximate PFW ratio of 1:4.5:12 and the fat source used was coconut oil. The method used was the same as for ice cream 4, however, while dissolving the sediment in the water, it was heated to only 60°C.

Ice cream 7 was made with the same approximate PFW ratio of 1:4.5:12 as ice cream 6, and with the same fat source, but the method was changed. Sediment and water were weighed and added to a saucepan. The mixture was mixed by hand whisking and heated to 60°C in order to disperse the proteins into the water phase. The sugar was added and allowed to dissolve. The mixture was then cooled in a water bath until it reached 30°C. The mixture was blended with a hand blender (Coline CW1299 Model LW-3318) for 2 min. The fat was added slowly while blending and the mixture was additionally blended form 1 min after the fat was added. The batter was frozen in an ice cream machine for 5 min until solid and the ice cream was stored in a freezer at -18°C.

The method used for ice cream 8 was the same as for ice cream 7. The same approximate PFW ratio of 1:4.5:12 was also used. The fat source was changed to have a coconut oil to rapeseed oil ratio of 1:1.

For ice cream 9, the method was the same as for ice cream 7 and with the same approximate PFW ratio of 1:4.5:12. The coconut oil to rapeseed oil ratio was changed to 1:2.

3.7.5 Analysis of the Ice Creams

Two evaluations were made on the ice creams. This was done by one person without training in sensory evaluation and the test was not standardized.

The first evaluation was made by tasting the ice cream to determine the mouthfeel by looking for creaminess and smoothness like the ice cream reference. This evaluation is termed “taste test”.

The second evaluation test the texture and the feel when dragging a spoon through the ice cream. This determines how solid and creamy it felt as compared to the ice cream reference. This evaluation was termed “the spoon test”.

No further analyses were made on the ice cream.

4 Results and discussion

4.1 Optimization of the Extraction of Hempseed Proteins

The experiments of optimizing the extraction of hempseed proteins from press cake can be analyzed in three different parts. Different pH treatments, extraction times and extraction scales were analyzed to find the parameters that produce a sediment with high protein purity in an efficient process with high yields.

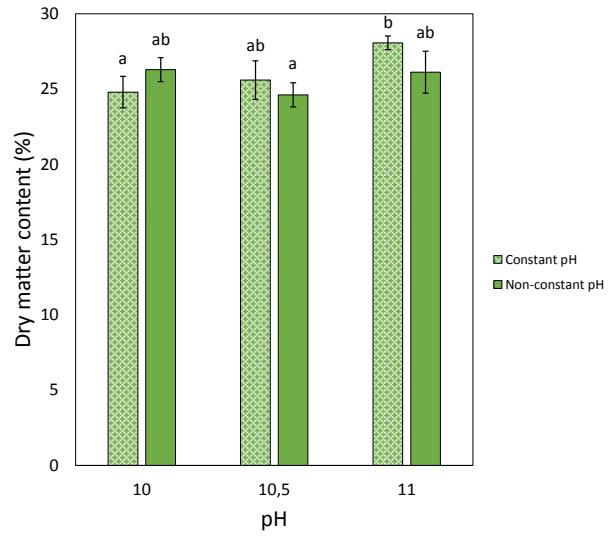
4.1.1 Different pH Treatments

The extraction step was tested with six different pH treatment (3.3.1). pH 10, 10.5 or 11 was used by either keeping the pH constant during the entire extraction time (constant pH) or adjusting the pH in the start of the extraction time (non-constant pH). The results for the non-constant pH treatments were obtained in collaboration with another study.

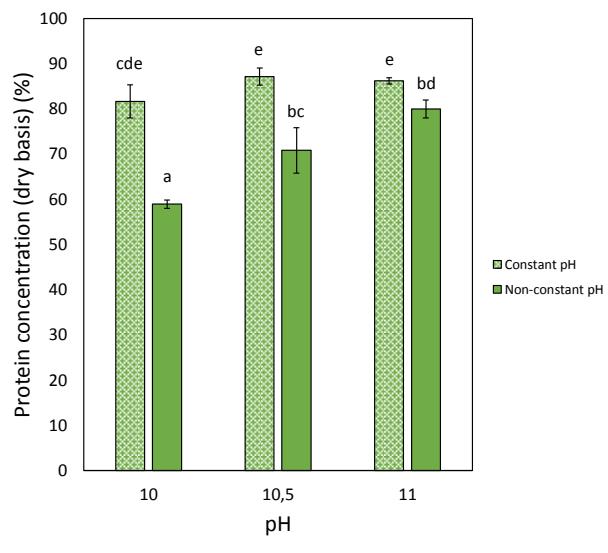
The dry matter content (Figure 4a) seem to be very alike for all the tested pH. A statistical analysis using UGLM was made to test the statistical significance. A constant pH of 11 gave the highest dry matter content, but there is no significant difference ($p<0.05$) to constant pH 10 and 10.5 or non-constant pH 11. Since there were many treatments that is not significant different, the dry matter content does not give any definitive indication of the most efficient pH to use.

The protein concentration in dry basis (Figure 4b) shows how much of the dry solids that constitutes of proteins. The protein concentration differed slightly between the different pH treatments. Since the data was not normally distributed, UGLM could not be used, so a t-test with two samples assuming equal variances was made for each treatment. Constant pH 10.5 gave the highest protein concentration on dry basis but there was no significant difference ($p<0.05$) from constant pH 10 and 11. However, non-constant pH 10 differed from non-constant pH 10.5 and 11. It can also be seen that for non-constant pH treatment, there was a clear increase of protein concentration in the sediment with increasing pH. However, no differences were found between the different pH for the samples treated with constant pH. In addition, the constant pH gave higher protein concentrations than their non-constant counterpart.

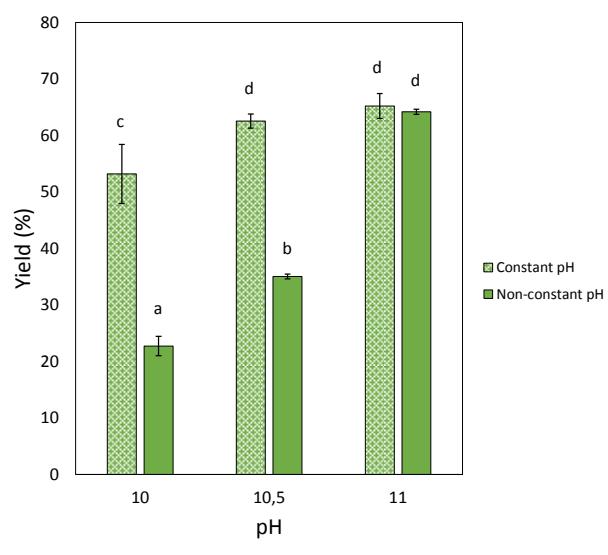
The yield (Figure 4c), was calculated based on how much protein was present in the sediment compared to in the press cake. The highest yield was obtained by constant pH 11, however, based on the UGLM, there was no significant difference ($p<0.05$) as compared to constant pH 10.5 and non-constant pH 11. The yield followed the same trend as the protein concentration where an increase in pH in the non-constant pH gave a higher yield. However, when keeping the pH constant, the yield almost doubled, indicating that it could be possible to use a lower pH if keeping it constant.



a)



b)



c)

Figure 4 a) The dry matter content (%) in the sediment, b) the protein concentration on dry basis (%) in the sediment and c) the yield (%) in the sediment for Constant pH 10, 10.5 and 11 and Non-Constant pH 10, 10.5 and 11.

The extraction coefficient (Figure 5) evaluates the efficiency of the treatment for extracting the proteins into solution. This by measuring how much of the proteins from the press cake that ended up in the liquid phase. The aim was to extract as much proteins as possible into solution to make them available for precipitation in the following step. The treatment with the highest extraction coefficient was pH 11, both constant and non-constant. There was however no significant difference between constant pH 11 and 10.5, and non-constant pH 11 according to UGLM. The extraction coefficient seems to behave in the same way as the yield, further strengthening the theory that a constant pH solubilizes more protein from the press cake than a non-constant pH.

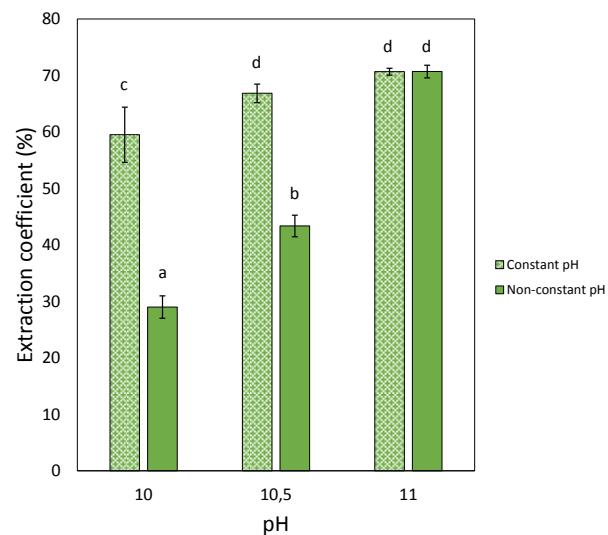


Figure 5 The extraction coefficient (%) in the sediment for Constant pH 10, 10.5 and 11 and Non-Constant pH 10, 10.5 and 11.

The precipitation coefficient (Figure 6) evaluates how efficient the precipitation step was. This was measured by how much of the extracted proteins in the liquid phase that could be precipitated into the sediment. The highest precipitation coefficient was calculated for constant pH 10.5 but according to the statistical analysis there was no significant difference ($p<0.05$) from constant pH 10 and 11 and non-constant pH 11. The precipitation coefficient also followed the same trend as the extraction coefficient, but the differences were visually smaller. This shows that the pH treatment during the extraction in the extraction step did not have as big impact on the precipitation as for the extraction, which was expected. For the constant pH treatments almost all of the protein that was extracted could be precipitated (over 90%).

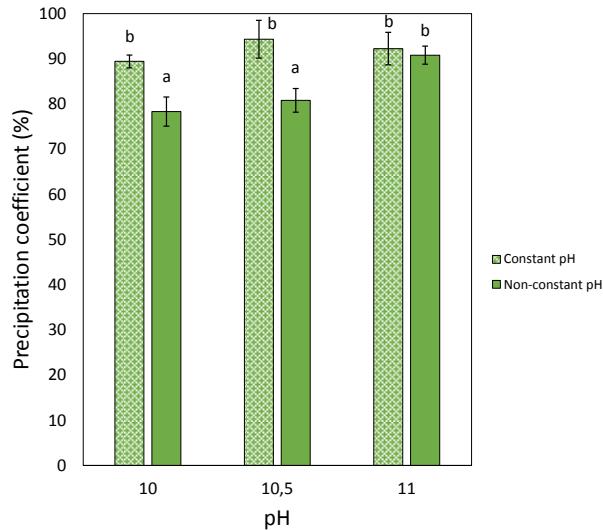


Figure 6 The precipitation coefficient (%) in the sediment for Constant pH 10, 10.5 and 11 and Non-Constant pH 10, 10.5 and 11.

Taking all the results into account, the constant pH 10.5 and 11 and the non-constant pH 11 gave overall the highest protein concentrations, yields, extraction coefficients and precipitation coefficients. A lower pH would be preferable to work with, both for safety aspects but also for less corrosion to the equipment. Because of this a constant pH 10.5 was preferred in the extraction step.

4.1.2 Different Extraction Times

Based on the result from the comparison between constant pH and non-constant pH (3.3.1), a constant pH of 10.5 was used during the extraction in the extraction step. Experiments were performed for extraction times of 1 h, 2 h, 3 h and 4 h, but because of lack of data due to the screening of 2 h and 3 h, they were not included in the statistical analysis even if the data was still included in the results.

From Figure 7a and 7b, it can be seen that 1 h and 4 h extraction time in the extraction step did not differ in either dry matter content nor protein concentration. The yield, based on amount of proteins in the press cake related to the amount protein in the sediment, in Figure 7c shows that there was no difference between 1 h and 4 h.

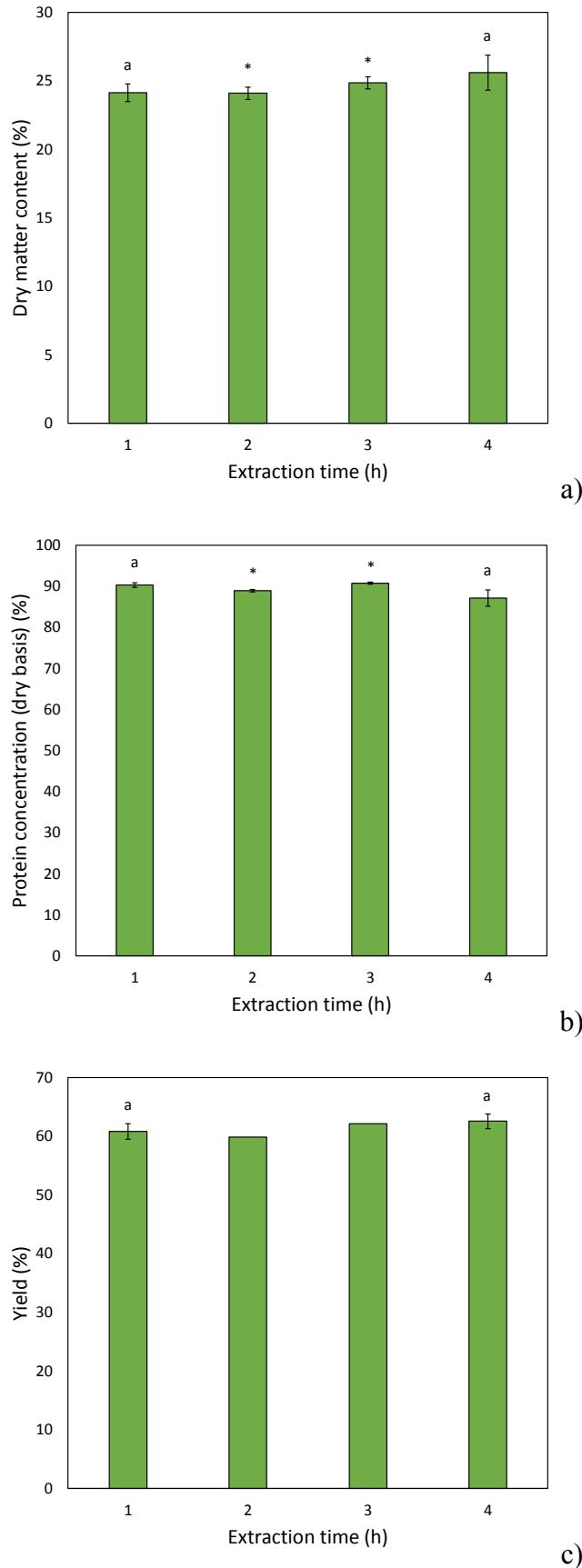


Figure 7 a) The dry matter content (%) in the sediment, b) the protein concentration (%) on dry basis in the sediment and c) the yield (%) for the different extraction times. *Was not included in the statistical analysis since only a screening was made.

From the analysis on protein content and dry matter content performed on the sediment and the solid phase, the efficiency of the extraction step and the precipitation step were calculated. Neither the extraction coefficient, Figure 8a, nor the precipitation coefficient Figure 8b, displayed any difference between 1 h and 4 h samples.

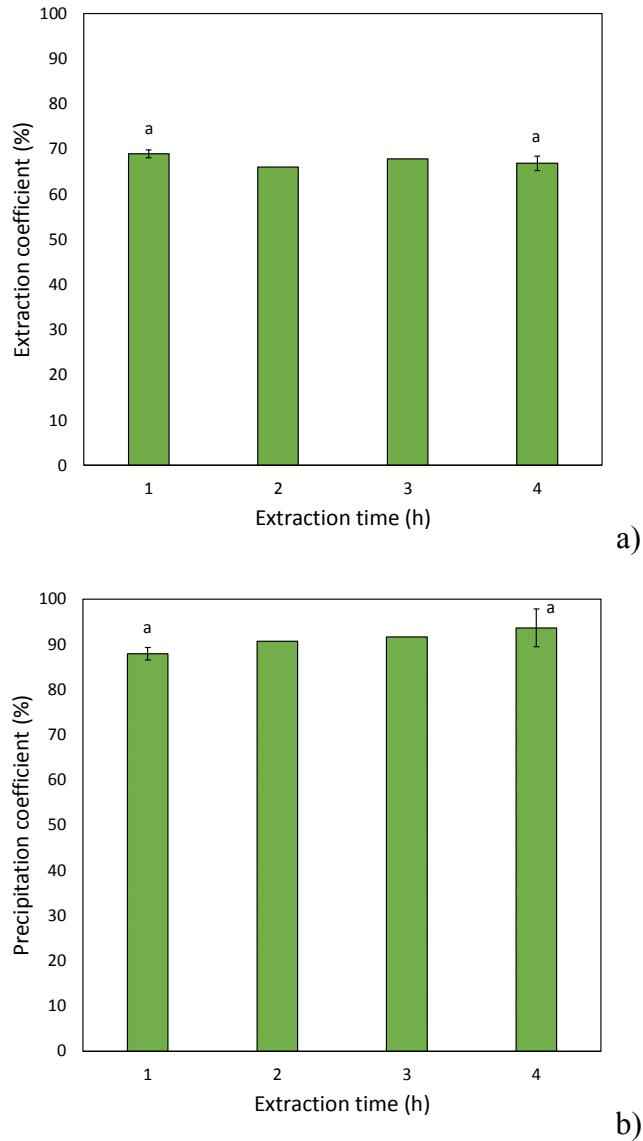


Figure 8 a) The extraction coefficient and b) the precipitation coefficient for the extraction times.

The extraction coefficient did not differ for 1 h and 4 h extraction times in the extraction step which indicates that the proteins were solubilized within the first hour of the alkali treatment. Therefore, having a longer extraction time would be unnecessary since no more protein would be solubilized. The precipitation coefficient did not decrease either when decreasing the extraction time to 1 h. This means that a shorter extraction time did not affect the proteins ability to be precipitated. This was further strengthened by the yield results and protein concentrations on dry basis not being decreased with decreasing time. However, nothing can be said about what happens within the first hour of the extraction and if the extraction time can be reduced further.

4.1.3 Comparison to Previously Done Studies

When comparing to the literature, the approximate same method for alkali extraction and isoelectric precipitation have been used. However, in the extraction step, pH 10.0 has been used as a non-constant pH, with varying extraction times (more than 1 h and 2 h) and extraction temperatures (35°C to 37°C). A different precipitation pH is also used at pH 5.0 and the extracted proteins have been resuspended and adjusted to a pH closer to 7 before being freeze dried (Tang et al, 2006; Malomo et al., 2014;).

The approximate same protein concentrations are achieved (85%) for both methods but different yield are reported. Tang et al. (2006) has a yield of 73%, calculated compared to the total protein content from the hemp seed meal that was used and Malomo et al. (2014) only achieves 38%. These differences could be due to numerous factors, including differences in the raw material that was used. Both methods produced a protein extract with the approximate purity as was obtained in this report, however the yield varied greatly and was both higher and lower than this reported value.

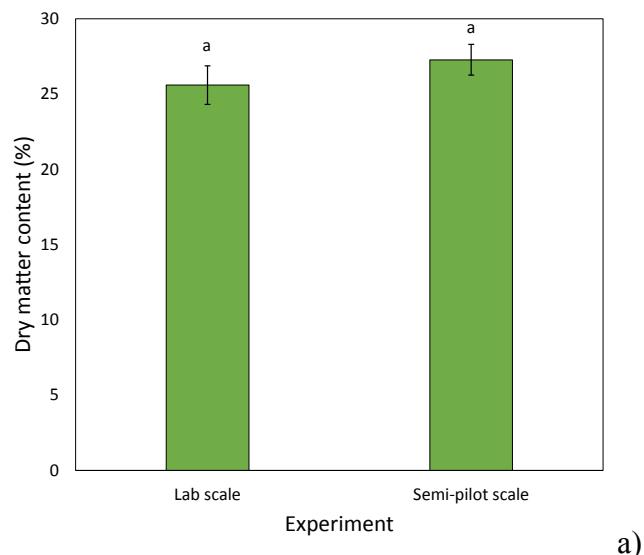
Wang et al. (2008) made a similar study, with the approximate same method of extraction. However, the extraction step was performed at room temperature with a 1 h extraction time with a pH adjustment to pH 10.0. The precipitation was made at pH 5.0 but at temperature 4.0°C. Before freeze drying the extracted sediment was adjusted to pH 7 and dialyzed at 4°C. This method produced a hemp protein isolate with a protein concentration of 90.5%. The protein concentration determined for this optimization is close to the reported value by Wang et al. (2008), even though no downstream processing of purification was made. This indicates that if keeping a constant pH, a larger amount of proteins could be solubilized and a high protein purity could be achieved without the purification, which would greatly reduce the costs in an industrial process.

4.1.4 Scaling Up to Semi-Pilot Scale

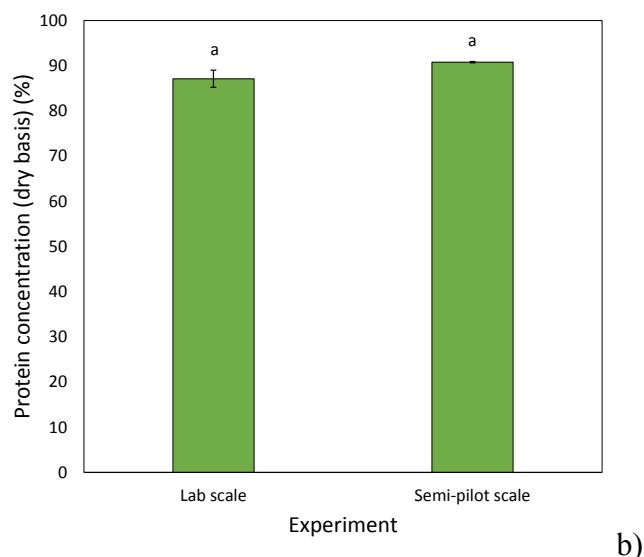
An extraction was made in semi-pilot scale in order produce material for the ice cream formulations. The same analyses were performed for the semi-pilot scale as for lab scale, so a comparison could be done between lab scale and semi-pilot scale, since both extractions were made with a constant pH 10.5 and 4 h extraction time in the extraction step. The data used for the lab scale was the same data as for the constant pH 10.5, with 4 h extraction in the extraction step (3.3.1.2). The reason for using 4 h extraction time and not 1 h was that the 4 h extraction time was tested in the equipment previously with a different material, making it possible to compare the materials in the future.

The dry matter content and the protein concentration (Figure 9a and 9b), show no difference between lab scale and semi-pilot scale. A t-test with two samples assuming equal variances showed no significant difference ($p < 0.05$) between lab scale and semi-pilot scale.

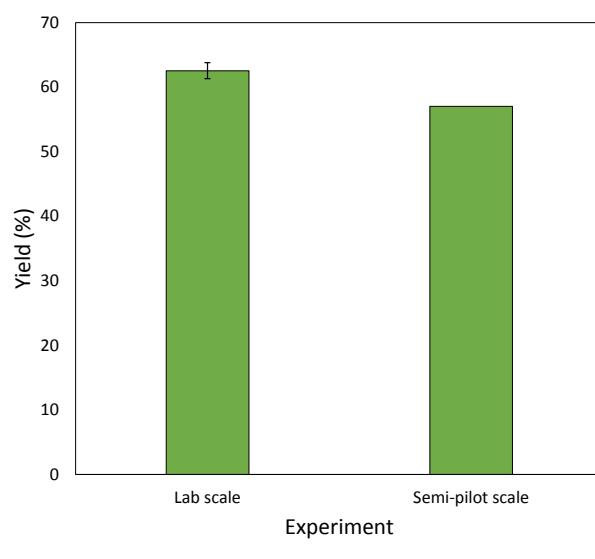
The yield (Figure 9c), however, seem to be lower for semi-pilot scale. No standard deviation could be calculated for the semi-pilot scale since the process was only run once, and it was thereby not possible to determine whereas there were any significant differences or not, but visually, it seems to be a difference



a)



b)



c)

Figure 9 a) The dry matter content (%) in the sediment, b) the protein concentration (%) on dry basis in the sediment and c) the yield (%) in the sediment for lab scale and semi-pilot scale.

The extraction coefficient (Figure 10) seems to be very similar for both experiments even without a statistical analysis. This indicates that the extraction might not be affected by the larger scale.

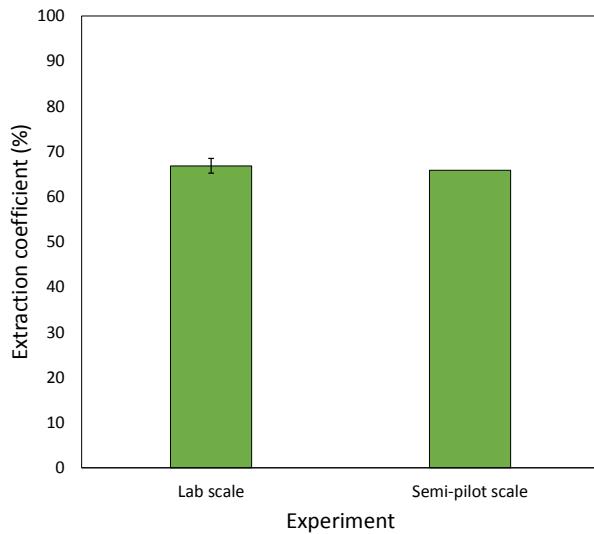


Figure 10 The extraction coefficient (%) for lab scale and semi-pilot scale.

From a visual evaluation of Figure 11, it was concluded that there was a difference between the precipitation coefficients from the lab scale and the semi-pilot scale experiments. The semi-pilot scale had a lower precipitation efficiency which could be related to separation step 1. In the lab scale, liquid phase and solid phase were separated in smaller batches using a centrifuge (3.3.1.2) and in the semi-pilot scale, the two phases were continuously separated by a decanter (3.3.3). A theory on why the precipitation coefficient was lower, e.g. why less of the extracted protein was precipitated, was that the decanter was not as good as the centrifuge in separating the liquid phase from the solid phase. Because of that, more non-nitrogen substances, like soluble fibers could have ended up in the liquid phase making it harder to precipitate the proteins.

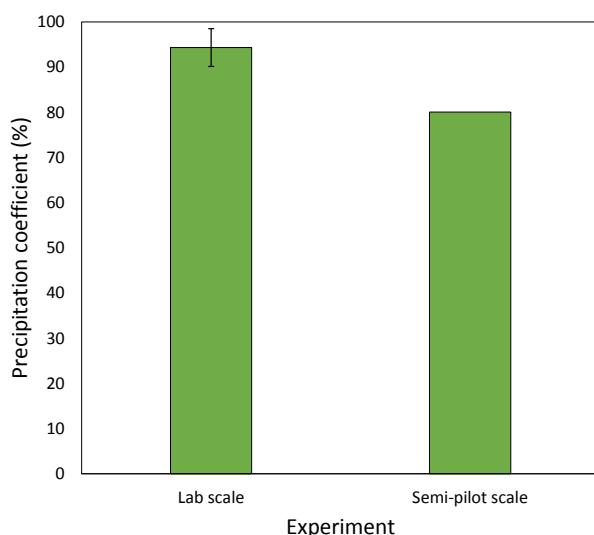


Figure 11 The precipitation coefficient (%) for lab scale and semi-pilot scale.

The results showed that it was possible to scale up the extraction process of hempseed proteins from press cake using an alkali extraction with isoelectric precipitation to a semi-pilot scale. It would be preferable to collect more data in order to evaluate the efficiency of the up-scaled process further.

4.2 Functional Analyses

4.2.1 Emulsifying Properties

From the particle size distribution measurements, three different means were given for each sample: the mode (Figure 12a), the volume weighted mean (d_{43}) (Figure 12b) and the surface weighted mean (d_{32}) (Figure 12c). Evaluating the results from the different means and the mode, there was a similar trend that with increasing protein concentration, the droplet size was decreased. This is reasonable, since an increasing amount of proteins will be able to stabilize a larger water to oil interface, which produces smaller droplets by counteracting coalescence. It can also be seen that when the protein concentration was increased above 8 mg protein/ ml oil, the emulsion droplet size did not decrease as rapidly for higher concentrations as it did for lower concentrations. This plateau is interesting, since it gave information about what protein concentration needed in order to create an emulsion and how small oil droplets the protein was able to stabilize.

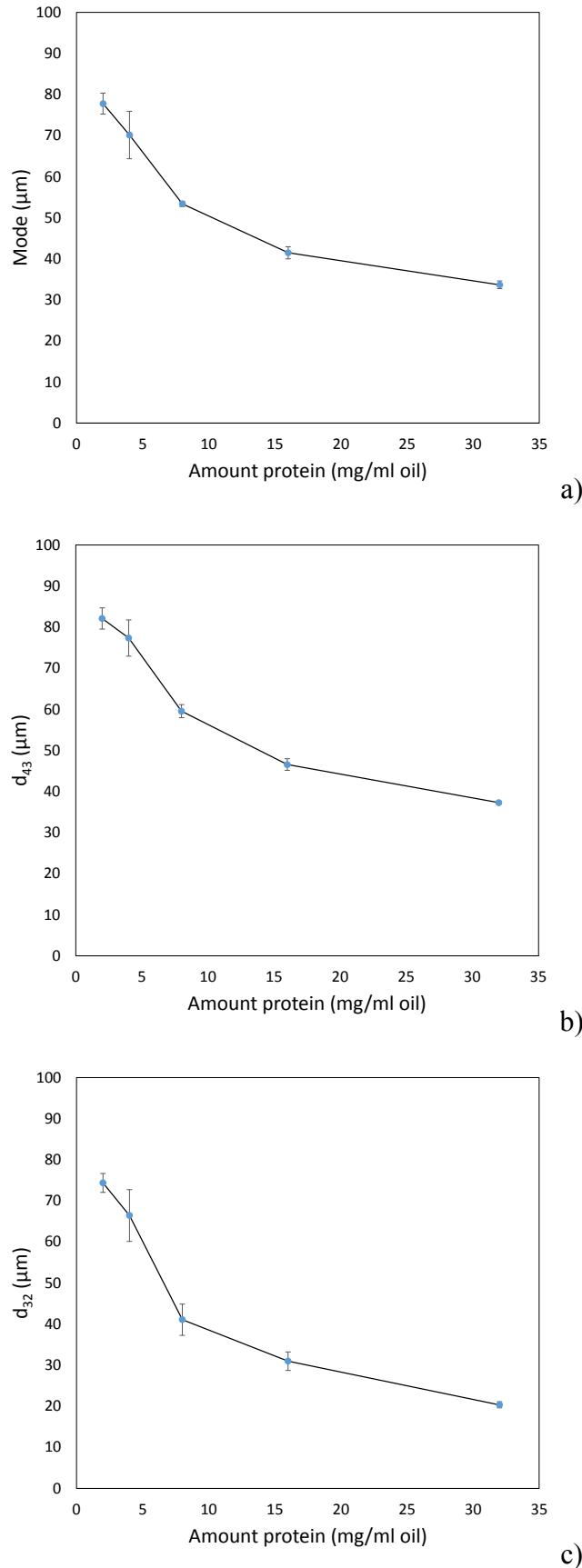


Figure 12 a) The mode, b) the volume weighted mean, d_{43} (μm) and c) the surface weighted mean, d_{32} (μm) for the particle size measurements on sediment.

In the oil-in-water emulsions made from the hempseed proteins, the most important destabilizing mechanism was coalescence, where the oil droplets merge to larger droplets due to a too small amount of proteins to cover the surface. Because of this, the volume weighed mean, (d_{43}), is more relevant to use in this case. Looking at the volume weighed mean only, the critical concentration of 8 mg protein/ ml oil, gave a d_{43} of approximately 60 μm . If the protein concentration was increased to 16 and 32 mg protein/ ml oil, the particle size decreased.

The results show that the hempseed protein extract can produce an emulsion and thus have an emulsifying ability. However, in order to evaluate how good emulsifying ability, the protein rich hempseed sediment had, it had to be compared to other proteins. In a master thesis report (Nilsson K., 2015), the same method for measuring the emulsifying properties of oleosin (a rapeseed protein), dried egg powder and egg white albumen was used. Rapeseeds contains a protein called oleosin that can be extracted with a method similar to the method used for extracting hempseed protein. Oleosin powders extracted at pH 9 from the thesis, gave d_{43} values of approximately 34 μm , 24 μm and 19 μm for the protein's concentrations of 8, 16 and 32 mg protein/ ml oil. Similar values for dried egg powder was reported. Compared to oleosin and dried egg powder, the hempseed sediment was not as good emulsifier agent as the above mentioned, since the droplet size in the emulsions produced with hempseed proteins were larger. Egg white albumen as emulsifying agent, produced oil droplets with d_{43} values of approximately 50 μm , 30 μm and 19 μm for the protein concentration of 8, 16 and 32 mg protein/ ml oil. The hempseed proteins have an emulsifying capacity closer to egg white albumen than oleosin.

It was also observed from the particle size distribution, that a higher protein concentration gave a lower emulsion droplet size (Figure 13). The smallest droplets appeared for 16 and 32 mg protein/ ml oil, and in addition a small peak below 10 μm was observed as well. This peak could be due to a too high concentration of protein, indicating that not all of the protein interacted with the oil-water interface but instead associated with each other, forming protein aggregates. This can also be related to the protein plateau mention above that formed at protein concentrations higher than 8 mg protein/ ml oil, where the larger amount of proteins did not contribute to a smaller droplet size but formed protein aggregates instead.

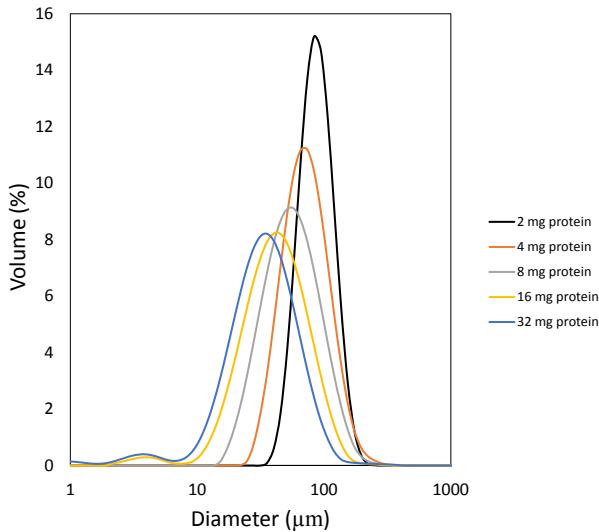


Figure 13 The particle size distribution for the emulsion made from hempseed proteins for different protein concentrations (mg protein/ml oil).

The reason for analyzing the emulsifying properties was partly to characterize the sediment, but also to evaluate if the proteins would work as emulsifying agents when producing oil-in-water emulsions, like ice cream. The sediment was able to stabilize an emulsion, indicating that it should be possible to produce an ice cream emulsion with hempseed proteins.

4.2.2 Differential Scanning Calorimetry

The freeze-dried sediment (3.4.1.2) was analyzed by DSC in order to evaluate if the proteins could go through any phase transitions during heating and how much energy it would require. Four replicates were analyzed, but unfortunately two of the replicates leaked during heating and had to be disregarded. The first sample showed a distinct peak with a peak temperature of 77.0°C and transition enthalpy of 6.37 J/g. Two other peaks could be detected with peak temperatures of 102.6°C and 120.4°C but these had significantly lower transition enthalpies than the major peak (0.45 J/g and 1.88 J/g respectively) (Figure A1 in appendix). The second sample showed a main peak with a peak temperature of 81.8°C and a transition enthalpy of 9.54 J/g. No peaks could be detected after the main peak as for sample one, but two smaller peaks before the main peak could be seen instead at peak temperatures of 52.1°C and 62.2°C but these also had smaller transition enthalpies (1.33 J/g and 0.29 J/g respectively) (Figure A2 in appendix). The smaller peaks both before and after the large peak, could be a result of other proteins or complexes that exists in the sample but in smaller quantities.

The samples that were prepared for the DSC measurements had been freeze dried, but not milled before analyzing. This resulted in a powder that was not homogenous, which could account for the differences in the results. However, both samples that were successfully analyzed did show a distinctive peak at 77.0°C and 81.8°C that could be attributed to Edestin, the main protein in hempseeds. Edestin have been reported to have a higher transition temperature when being examined by DSC, which is attributed to its denaturation temperature of around 92°C to 95°C with onset temperatures at approximately 85°C. This when being measured from 20°C to 110° with a temperature incline of 5°C/min with pH 7 buffer (Tang et. al., 2006; Wang et. al. 2008).

Due to the differences in measurement methods, the results for transition temperatures will differ. The sediment samples were measured with a lower temperature incline of 10°C/min and without buffer. This could produce a phase transition at lower temperatures since the proteins had more time to transition. It is also possible that different varieties of hemp differ in protein composition and characteristics, and since it is not known what type were used, differences in results could be attributed to this.

4.2.3 Rapid Visco Analyzer

Hemp protein powder with protein concentrations of 35%, 50% and 70%, and freeze-dried sediment were examined in a rapid visco analyzer in order to evaluate its gelling behavior. The commercially produced hemp protein powders were able to form gels (Figure 14). All three protein powders showed a cold-swelling ability, with a viscosity peak of approximately 100 cP. For 70% protein, there was a clear viscosity peak. For the lower concentrations, the viscosity peak decreased, with only a small peak for 50% and no peak could be detected for 35%. Since the peak size increased with increasing protein concentration, it indicated that it was the proteins that were responsible for the viscosity change.

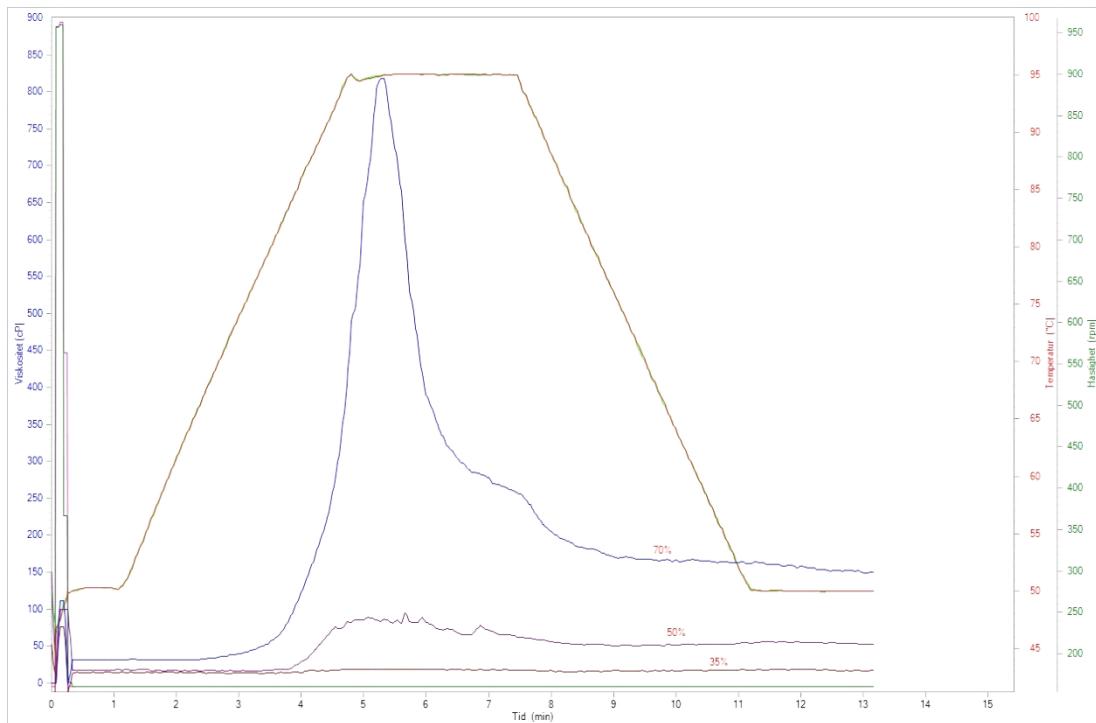


Figure 14 The results from the RVA measurement on bought hemp protein powder with the concentrations 70% (blue), 50% (purple) and 35% (red). The different concentrations are also marked in the figure.

The freeze-dried sediment was analyzed with both 3.5 g and 4.5 g sample. No heat induced viscosity peak could be seen at 3.5 g sample, which was why the sample amount was increased (Figure 15). Unfortunately, neither of the samples showed a viscosity peak when heat was applied. They did, however, show a cold swelling peak at approximately 100 cP, similar to the protein powders.

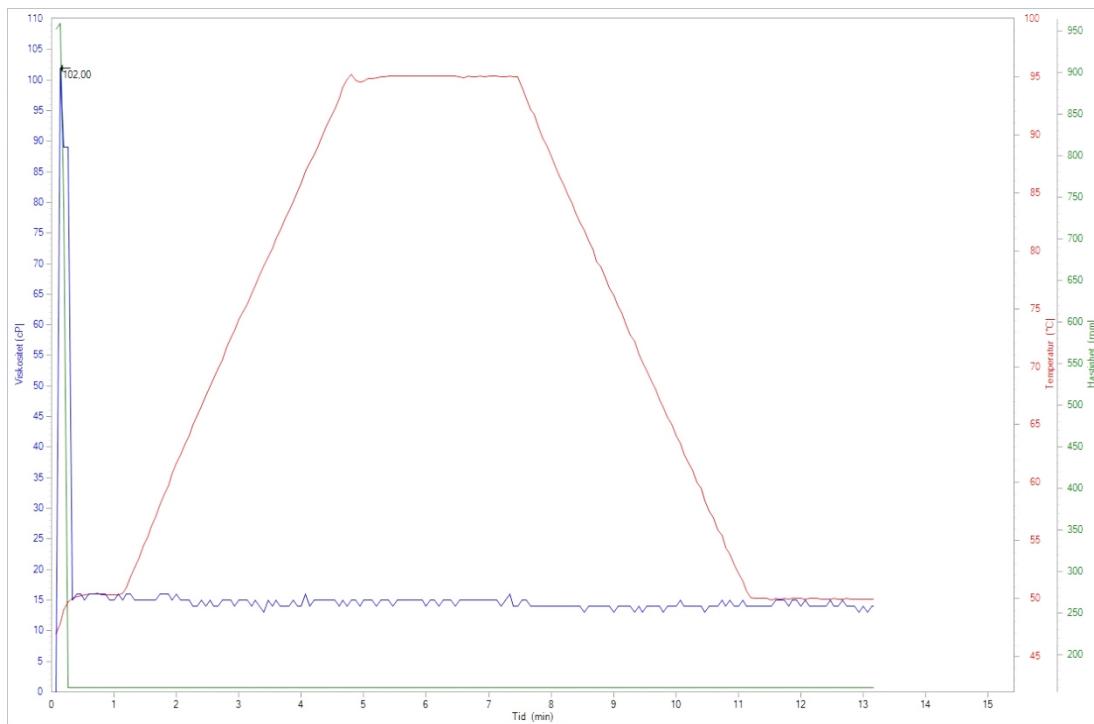


Figure 15 The results from the RVA measurements on freeze dried sediment with 4.5 g sample.

There are a few theories of why the protein powders gave a viscosity increase and the sediments did not. The sediment had a protein concentration of approximately 90% on dry basis. It could be a too high protein purity in order for the sediment to be able to form a gel network. With the lower protein concentrations of the protein powder there could be more non-protein materials that aids in the gel formation. It could also be that there was an optimal ratio between proteins and non-protein material needed in order for the hemp proteins to form a network. This concentration could be between 90% and 70%, but it might also be between 70% and 50%.

Another study has been done by Malomo et al. (2014) where the proteins gelling ability was examined. This by mixing 2% to 20% w/v on a protein basis in water and heating the mixture to 95°C for 1 h. The mixture was then cooled and stored in 4°C for 14 h. This showed that the hemp protein isolate had poor gelling abilities. These studies were however performed with a different gel forming method, but it strengthens the conclusion that hemp proteins are poor at forming networks. It was also unknown how the hemp protein powders had previously been treated during production. It could thus be hard to evaluate the proteins ability to form a network in comparison to the protein powder. The alkali extraction with isoelectric precipitation could have impacted the proteins ability to form a network by decreasing its molecular flexibility as was also suggested by Malomo et. al (2014).

4.3 Ice Cream Formulation

In the ice cream experiment, nine different ice creams were made, whereas one reference, two hemp heart ice creams and 6 formulations based on extracted sediment.

The ice cream reference, ice cream 1, was a cream and egg yolk-based ice cream with a vanilla flavor (Figure 16). There are two characteristics of the ice cream reference that will be used to evaluate the ice cream formulations. First, it had a very smooth and creamy mouthfeel when

being tasted (the taste test). Second, it had a solid but creamy texture, without crumbling, when dragging a spoon through the ice cream when it came directly from the ice cream machine (the spoon test).



Figure 16 Ice cream 1, the ice cream reference.

The next ice cream formulation was based on dehulled hemp hearts, since recipes for this was found during the literature search. Two flavors were made, the first hemp heart ice cream, ice cream 2, was a vanilla flavor (Figure 17a). It had a slightly creamy mouthfeel but left a very oily taste and mouthfeel afterwards. The mouthfeel was not very smooth, which probably was due to the shells not being mixed entirely but had some shell residue on them. The taste of the hemp heart ice cream with vanilla was very strong, and bitter. This could be because of the strong flavor of hemp oil and also the bitterness of the shells of the hempseeds. The texture when performing the spoon test was similar to the ice cream reference in creaminess but it was not as solid as the reference.

The hemp heart ice cream with chocolate flavor, ice cream 3, (Figure 17b), had in general the same mouthfeel, feel from the spoon test and look as the hemp heart ice cream with vanilla flavor. It had somewhat grainier mouthfeel, but this could be due to the blender becoming very hot while mixing the hempseeds, and they were not mixed for as long time as ice cream 2. The texture when performing the spoon test was similar to the ice cream reference, creamy and solid. When flavoring the hemp heart ice cream with chocolate and salt instead of vanilla, a lot of the bitter flavor disappeared, but it was not hidden entirely. The oily feeling after tasting the ice cream was still present.



Figure 17 a) Ice cream 2, the hemp heart ice cream with vanilla flavor and b) ice cream 3, hemp heart ice cream with chocolate flavor.

The next ice cream formulation was based on the recovered protein rich sediment. The first sediment was produced in a semi-pilot scale to produce enough material for the ice cream formulation. The sediment had been stored in plastic containers in the freezer at -18°C and thawed before using in the ice cream. Two ice creams were made with the same protein to fat to water (PFW) ratio of 1:4.5:19, approximately the same ratio as the ice cream reference. However, two different fat sources were used, one with hemp oil, which is ice cream 4 (Figure 18a), and one with coconut oil, ice cream 5 (Figure 18b). The first observation in making the ice cream was that the sediment was grainy. A theory is that the protein has aggregated and separated from the water phase. Dispersing the proteins in the water during heating was unsuccessful, giving the ice cream a very grainy mouthfeel after freezing it in the ice cream machine. The texture of both ice creams was very icy and more of a slushy like texture that was unable to hold together than a creamy texture. Using different fat sources in the ice creams did not give a very big difference in texture. The coconut oil gave a slightly more solid feel in the spoon test than the hemp oil. The hemp oil, however, gave a very strong flavor that could be hard to disguise. A theory of why the texture became icy, is that the proteins were unable to go into solution, and thus not available for the emulsion formulation.



Figure 18 a) Ice cream 4, sediment based ice cream from frozen sediment, a sediment to fat to water ratio of 1:4.5:19 and hemp oil as fat source and b) ice cream 5, sediment based ice cream from frozen sediment, a sediment to fat to water ratio of 1:4.5:19 and coconut oil as fat source.

To avoid the issue with the aggregated proteins creating a grainy texture, freshly produced sediment was used instead. First an ice cream based on fresh sediment with a sediment to fat to water ratio of 1:4.5:12 was made with the same method as the previous ice creams, ice cream 6 (Figure 19a). When changing to a fresh sediment, the issue with a grainy product disappeared and the mouthfeel became much smoother. When decreasing the amount of water, the ice cream texture became much less icy, it reminded more of a sorbet in mouthfeel but still not creamy like the ice cream reference. When doing the spoon test, it still felt a bit like sorbet, and it had some trouble holding together.

The next step in the formulation development was to mix more vigorously when adding the fat in order to incorporate more air into the emulsion to make it creamier. The same ingredients and ratios were used but a hand blender was used instead of a hand whisk when adding the fat producing ice cream 7. This produced an ice cream that was much more solid and creamier than the previous ice creams (Figure 19b). The mouthfeel was very creamy and smooth, and the spoon test showed a very firm and creamy ice cream. It was however a bit too solid. A reason for this could be that the fat source constitutes of only coconut oil.

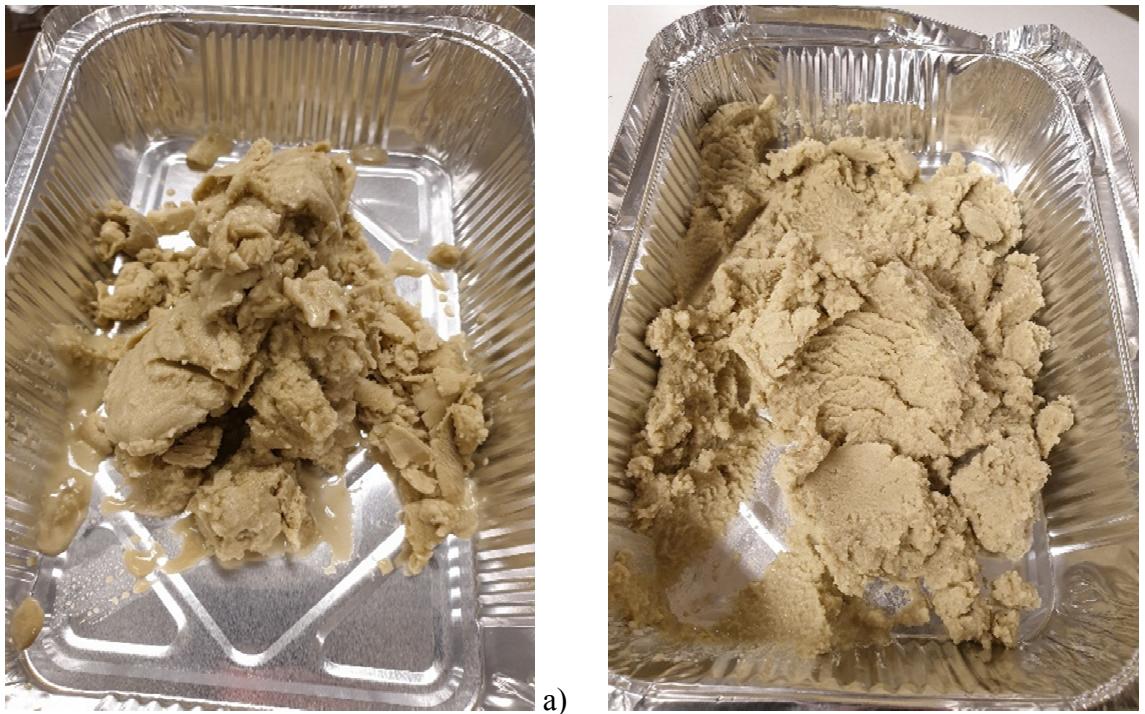


Figure 19 a) Ice cream 6, sediment based ice cream from fresh sediment, a sediment to fat to water ratio of 1:4.5:12 and coconut oil as fat source and b) ice cream 7, sediment based ice cream from fresh sediment, a sediment to fat to water ratio of 1:4.5:12 and coconut oil as fat source. Mixed with a hand blender.

To make the ice cream less firm but still creamy, the fat source was changed to have equal amounts of coconut oil and rapeseed oil. For ice cream 8, the same sediment to fat to water ratio of 1:4.5:12 was used and the fat was mixed in with a hand mixer. This produced an ice cream that both in the mouthfeel evaluation and the spoon test was very similar to the ice cream reference (Figure 20a). It had a very creamy and smooth mouthfeel and a solid and creamy texture.

To evaluate how a different ratio of coconut oil to rapeseed oil were going to change the texture, an ice cream with the coconut oil to rapeseed oil ratio of 1:2 were produced as ice cream 9. It gave similar qualities as the ice cream with a coconut oil to rapeseed oil ratio of 1:1 (Figure 20b). It was however, not holding together as well as the other ice cream, it crumbled much more.



a)



b)

Figure 20 a) Ice cream 8, sediment based ice cream from fresh sediment, a sediment to fat to water ratio of 1:4.5:12 and a coconut oil to rapeseed oil of 1:1 as fat source. Mixed with a hand blender. b) Ice cream 9, sediment based ice cream from fresh sediment, a sediment to fat to water ratio of 1:4.5:12 and a coconut oil to rapeseed oil of 1:2 as fat source. Mixed with a hand blender

Evaluating all the produced ice creams, the composition of sediment to fat to water that gave the creamiest ice cream was 1:4.5:12 with equal amounts of rapeseed oil and coconut oil. And the best results were obtained when mixing the emulsion vigorously using a blender to incorporate more air into the emulsion.

5 Conclusion

Three different experiments were made in the optimizations of the extraction of hempseed proteins. For the different pH treatments, the most optimal one was concluded to be constant pH 10.5. This since it had the highest yield, protein purity and efficiency of the process, while it was not as alkali as pH 11 and therefore better in a safety aspect. When it comes to the different extraction times, shorter extraction times was as efficient as the 4 h extraction. Because of this, the extraction time could be decreased to 1 h. A semi-pilot scale was also tested, however, only one test was made, and no definite conclusion could be made whether it was less efficient as lab scale or not. It did show, however, that it is possible to extract hempseed proteins with the alkali extraction and isoelectric precipitation in a semi-pilot scale.

Characterization of the proteins showed that it was possible to use the extracted hempseed proteins as an emulsifier. The RVA measurements gave inconclusive results since no viscosity change could be seen. The DSC measurements showed that a thermal transition happened around 80°C, which was identified as the main hempseed protein, Edestin.

The results that the hempseed proteins could work as an emulsifier were confirmed in the ice cream experiments. The different ice cream formulations that were tested yielded in one formulation with results similar to the ice cream reference. It was a PFW ratio of 1:4.5:12 with equal amounts of rapeseed oil and coconut oil as well as fresh sediment.

The overall conclusion is that a 1 h extraction at a constant pH 10.5 gave the most optimal results. It is also possible to extract hempseed proteins in a semi-pilot scale. Furthermore, hempseed proteins do have an emulsifying ability and to formulate an ice cream with the proteins is possible.

6 Future Work

Along with this project many new questions were discovered, but because of limitations with time and scope they could not be investigated. Firstly, the optimization of the extraction step of the protein isolation was thoroughly examined. However, only one replicate was made in a semi-pilot scale. In order to conclude whether the process is efficient enough to be run in a larger scale more test should be done. Also, a 1 h extraction time was tested to not be significantly different from the 4 h extraction time, but this have not been tested in the semi-pilot scale. Meaning, more test should be done in semi-pilot scale in preparation of scaling up the process.

When it comes to the characterization of the protein, more investigation is needed on the gelling ability since the sediment showed no gelling ability whereas the commercial protein powders did. No conclusion could be made on the proteins gelling ability or if the protein needs non-protein substances to be able to form a self-supporting network.

Another major issue that was discovered was that the sediment, when having been frozen and thawed, became grainy. A theory of why this happens is that the proteins dehydrates and aggregates. Tries to re-solubilize the proteins with the help of heat and whisking by hand was unsuccessful. Perhaps it would be possible to re-solubilize the proteins if mixing it more vigorously, using a blender of some sort. Otherwise more investigation of the freezing stability of the proteins need to be done, in order to determine what happens when the sediment is being frozen. At this stage, the sediment would have to be used immediately in the ice cream production since it lacks a good freezing stability, and shelf-life of the fresh sediment is unknown.

When it comes to the ice cream formulations, the recipes tested was quite simplified in order to evaluate the proteins ability to stabilize a frozen emulsion as an ice cream. The ice cream is not a finished product at this state, so more experiments need to be performed. For instance, the effect of adding flavors and sugar to the ice cream should be tested. The flavor profile of ice cream 8, that was the most successful one, was quite neutral, since the sediment had a very weak flavor. The main flavor that could be detected was the coconut oil, that should be possible the hide. Also, no stabilizers were used in the experiments. It has not been investigated how the ice cream behaves during storage in the freezer and if stabilizers are needed. The last issue with the ice cream was that the process was not adapted to a larger production. The recipe and the process have to be adapted in order to make an industrial processed hemp-based ice cream. Also, further texture analysis should be done on the ice cream since there is no knowledge of how the ice cream looks or behaves except for the taste test and spoon test that were performed.

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8 Appendix

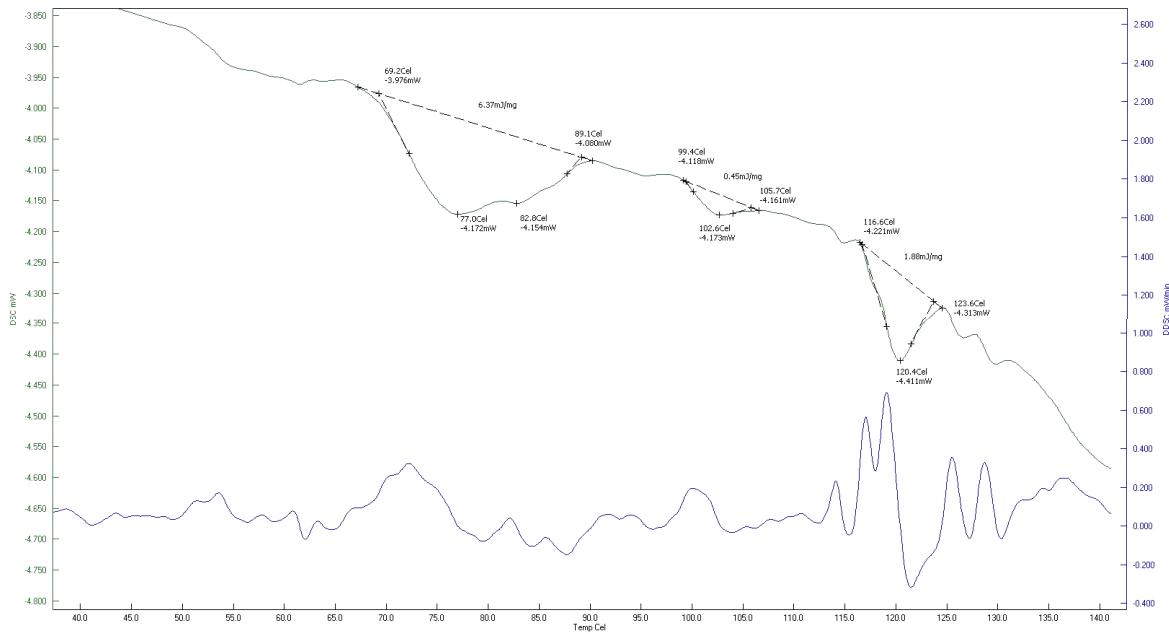


Figure A1 The DSC thermogram for sample 1 of freeze-dried sediment.

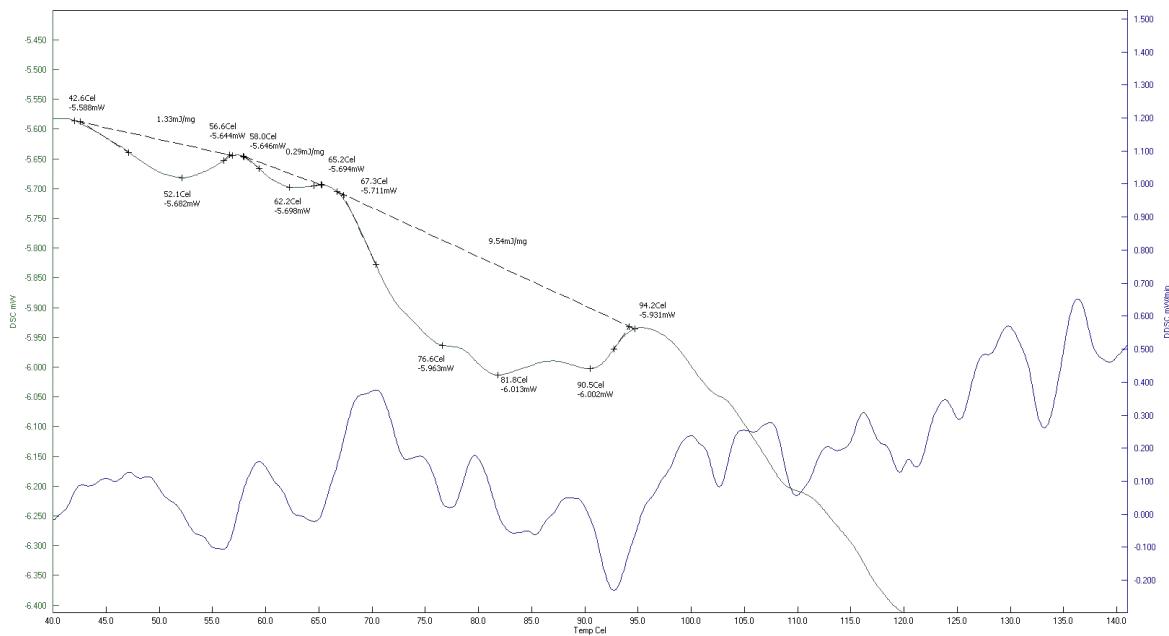


Figure A2 The DSC thermogram for sample 2 of freeze-dried sediment.