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MSc Thesis Biobased Chemistry and Technology

AQUEOUS EXTRACTION OF OIL BODIES AND PROTEINS FROM TOMATO PROCESSING RESIDUES

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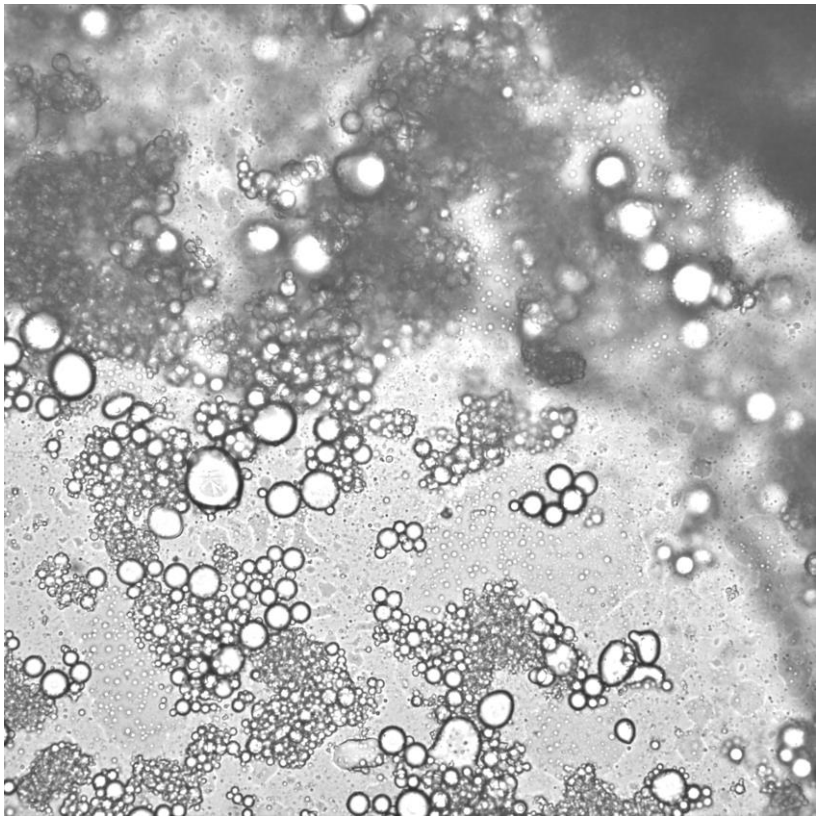

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AQUEOUS EXTRACTION OF OIL BODIES AND PROTEINS FROM TOMATO PROCESSING RESIDUES

An Assessment of Parameters and Characterization of the resulting Oil Body Cream

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ABSTRACT

Processing of tomatoes into products such as sauce, puree or ketchup creates a waste stream of tomato seeds and peels with an estimated volume of 1.63 Mt annually. Lack of suitable and scalable extraction methods hamper valorization of its components to-date. The potential of a mild aqueous extraction to obtain high-quality oil bodies and proteins from tomato processing residues is investigated. A set of extraction parameters (particle size, pH, solid:liquid ratio, salt concentration) was tested. Oil bodies could be purified from tomato seeds as a cream that contained 23.93±4.87% moisture, 61.01±0.97% oil and 0.82±0.14% protein but showed little stability when being stored at room temperature. An additional washing step did not increase the oil content (20.49±1.87% moisture, 50.63±3.48% oil and 0.29±0.07% protein) but resulted in improved stability that was attributed to a change in pH and less enzymatic activity. Extraction of tomato seed meal in 1:6 (w/v) demineralized water at pH 9 yielded 17.31% oil enclosed in oil bodies, while roughly 98% of the oil and 62% of the protein was removed from the raw material. Losses are therefore mostly attributed to suboptimal process conditions. Application of the extraction parameters determined for seeds on a mixed fraction of tomato peels and seeds did result in few unstable oil body flakes only, which could not be purified for analysis. Simultaneous aqueous extraction of oil bodies and proteins from tomato seeds is concluded to be a promising first step in a biorefinery cascade, that may be continued with a valorization of fibers.

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Appendix

1 INTRODUCTION

By 2050, the world population is expected to exceed 9 billion people, with a chance of even surpassing 10 billion[1–3]. Meeting the growing food demand for an increasingly wealthy population has been described as one of the most significant challenges in the 21st century[4]. It requires more efficient use of the current resources as well as the valorization of nutritional components from currently unused streams.

Supply of proteins providing a good ratio of amino acids is key to balanced nutrition, and its demand in 2050 is estimated to be 30-50% above current levels[5–8]. Thus, alternative protein sources for direct human consumption need to be explored[2,4,5]. For novel protein sources to be a valid alternative to the existing ones, new value chains need to be created during which various aspects such as food safety, scalability, and consumer acceptance should be taken into account[5,6].

Next to proteins and carbohydrates, vegetable fats and oils are an essential part of human nutrition[9]. While plenty of oil sources are available, awareness regarding their health impacts, e.g., a high content in unsaturated fatty acids and antioxidants, is increasing. Key parameters to assess the quality of vegetable oil are the quality and freshness of its raw material, its stability, clarity and shelf life[10]. For vegetable oils that are high in unsaturated fatty acids, it is essential that the quality of those remains high throughout the extraction process. Oxidation of the unsaturated fatty acids throughout the extraction process and subsequent storage should be avoided as far as possible.

1.1 WASTE STREAMS AS A NOVEL SOURCE OF OIL AND PROTEIN

Traditionally, industrial food processes are designed to obtain one single ingredient, which is mostly used as part of a more complex product formulation[11]. This design creates large waste streams containing many functional and nutritious components, such as biopolymers and bioactive compounds[11,12].

For example, oils are currently pressed or extracted from oilseeds and crops which are cultivated for the single purpose of oil production. Despite being rich in nutrients, the remaining press cake is poorly valorized[13]. Other agricultural products, such as vegetables and pulses, satisfy the bulk of global protein demand[5]. In their background paper, Swiss company Bühler argues that up to 50% of the additional protein required until 2050 could be sourced from current waste streams[6]. The poor availability of proteins inside these waste streams makes their use complex, which might be overcome if they could be used in their native matrix[12].

Biorefinery is defined as a concept to convert biomass into food and feed, fuels, power and value-added chemicals from biomass[14,15]. The application of this concept to agro-industrial waste streams has been suggested, and second-generation biorefineries have been sketched out for sugar beet pulp and molasse as well as grape and tomato pomace[13].

1.2 TOMATO RESIDUES FOR A SECOND-GENERATION BIOREFINERY

24% of the worldwide tomato production are not consumed freshly but processed further into tomato-based products[16]. Tomatoes can be processed into juice, pulp, and purée; they are used to prepare sauce, soup, ketchup, and dips and form a vast ingredient of ready-made meals such as pizza, lasagna, and stews. Most of these end products require slightly adjusted processing steps, but all of them include peeling of the ripe fruits[17]. For diced tomato cubes, pulp and juice, the tomatoes are also (at least partly) deseeded for a smoother textural feeling.

1.2.1 WASTE ACCUMULATION DURING TOMATO PROCESSING

Unused tomato seeds and peels leave the tomato processing facility as a mixed side stream, which is called *tomato pomace* and is depicted in Figure 1-1[13,18,19]. Depending on the variety to be processed, the processing equipment and the aimed product, this stream accounts for about 3-7% of the original fruit weight[13,20–23]. Currently, there are few applications available for waste streams such as tomato processing residues: to a limited extent it is fed to cattle[18,24,25] or is used for biogas production (personal communication, Mutti S.p.A.)[11]. Application of dried tomato pomace as a fertilizer has been suggested[26,27]. What cannot be eradicated via these processes before it perishes is commonly being transferred to landfills, which has been related to environmental problems as well as financial burdens for the processing companies[11,21,24,26,28].

Figure 1-1. Tomato pomace from a private tomato sauce production (Province of Salerno, Italy. July 2017)

According to EU Legislation, tomato pomace is considered a lower value side product suitable for feed applications[29].

1.2.1.1 Composition

The exact composition of the tomato seeds and peels used in this study remains to be elucidated. Previous studies reported tomato seeds to contain 18.0-28.1 wt.% oil and 24.5-39.3 wt.% protein[20,25,30]. The peels were distinctively poorer in both components, with 3.1-6.1 wt.% oil and 10.0-13.3 wt.% protein[20,25,31].

The discrepancies between the different publications can be explained different origins of the tomato seeds and peels, different years of harvest and various methods used to determine lipid and protein contents.

1.2.1.2 Previous valorization attempts

Former research has especially assessed the options to extract small amounts of useful components from tomato processing residues[32]. For example, tomato peels are rich in carotenoids (lycopene and α-, β- and γ-carotene), renown as potent antioxidants as well as for their function as a precursor of vitamin A[22,32–34]. The selective extraction of these high-value components will, however, not be part of this thesis.

Bulk product extraction was focused on tomato seeds, as they are richer in oil and proteins than the peels.

OIL FROM TOMATO SEEDS

As a first attempt to valorize tomato residues, tomato seed oil was produced as early as 1901 and was regularly mixed with olive oil in the 1960's, mostly as a part of the olive oil fraud[28,35]. Due to rapid oxidation of the oil, resulting in an unpleasant off-flavor and taste, production was soon shut down[28]. Later laboratory studies could not find evidence of extended oxidation compared to other types of vegetable oils[36]. As studies on a laboratory scale are generally monitored in more detail, oxidation after industrial production must still be assessed as a potential risk.

Regarding properties, tomato seed oil has been described as being similar to cottonseed and soybean oil[33,35,37]. Culinary purposes, as a salad oil or for the production of margarine, have been suggested, as well as cosmetic applications[35,38–40]. However, in 2017 tomato seed oil was only available from a few

countries (e.g., Austria, England, and the United States)[39–41]. The high price (> 100€/L) did not allow for its daily use.

As can be seen in Figure 1-2, tomato seed oil is rich in unsaturated fatty acids, especially linoleic and oleic acid. Linoleic acid is a polyunsaturated ω-6 fatty acid and oleic acid a monounsaturated ω-9 fatty acid. Linolenic acid, the only ω-3 fatty acid present, maximally constituted 2.31 wt.% of the total volume in the reviewed publications[42]. Palmitic acid accounts for the bulk of the saturated fatty acids.

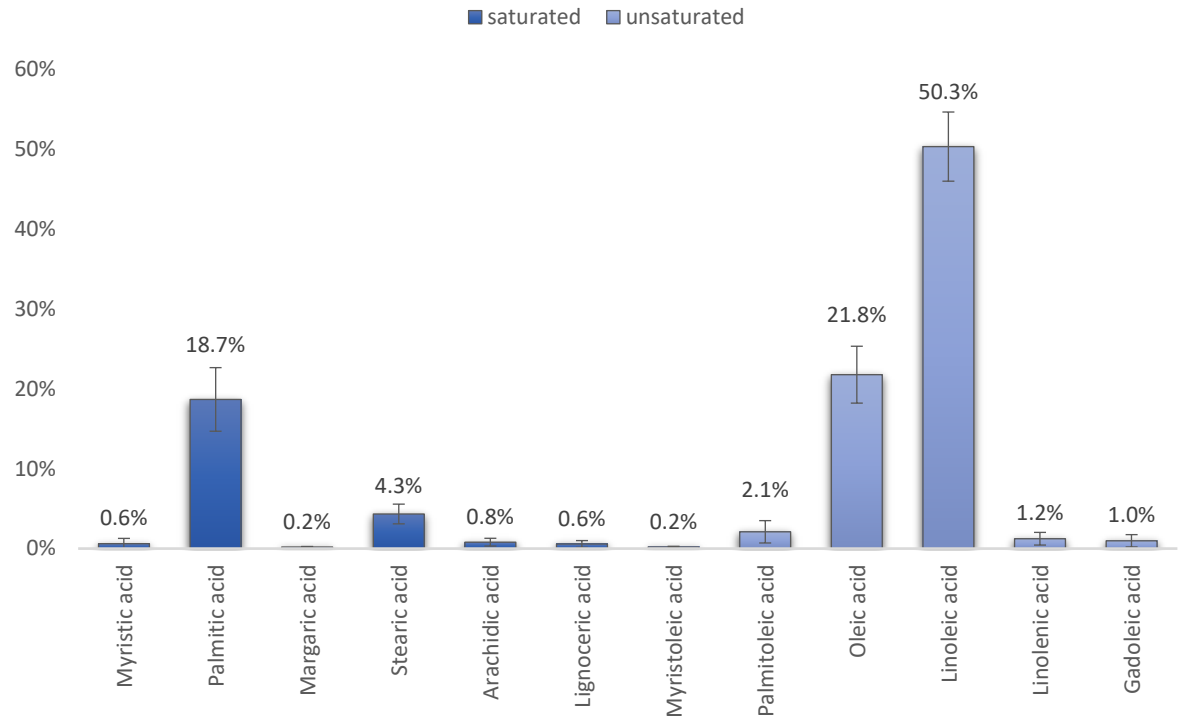

Figure 1-2. The approximate fatty acid profile of tomato seed oil. Bars show the average and standard deviation when values from Tsatsaronis & Boskou (1972)[43], Demirbas (2010)[44], El-Tamimi et al. (1979)[42], Lazos, Tsaknis and Lalas (1998)[45] and Rossini et al. (2013)[46] were taken into account. Only fatty acids with a concentration > 0.1% are displayed.

Ω-fatty acids are generally considered prone to oxidation. The high content of this type of fatty acids might have been the reason for the development of off-flavors described earlier. Encapsulation of fatty acids in oil bodies is expected to improve the oxidative stability[47,48].

Oil bodies (also called oleosomes) are the lipids-storing organelles in seed tissues and have been described for a variety of species, such as rapeseed, mustard, cotton, flax, peanut, sesame, olive, avocado, and maize[47,49–52].

Their structural properties are very similar across species[51]: a water-insoluble core of triacylglycerides is surrounded by a natural membrane of phospholipids and low molecular weight proteins (see Figure 4-5). The most abundant protein is called oleosin and varies in size in a range of 15-30 kDa[53].

The negatively charged oil body surface provides optimal chemical stability and prevents coalescence by steric hindrance and electric repulsion[50,53].

To our best knowledge, oil bodies they have not yet been extracted from tomato seeds, and their properties have consequently never been described.

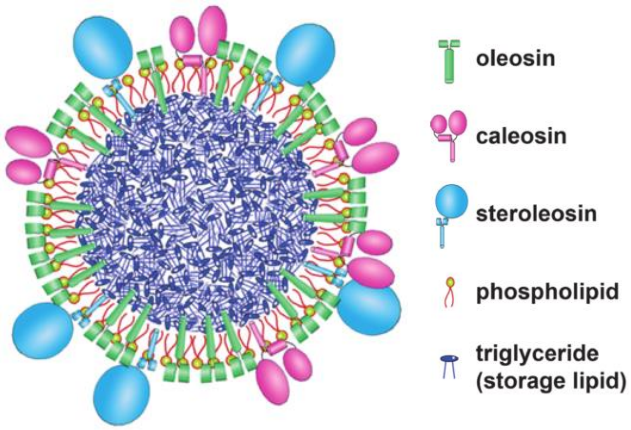

Figure 1-3. Mature oil body surrounded by oil body proteins (oleosin, caleosin and steroleosin). Graphic adjusted from Shimada and Hara-Nishimura (2010).

PROTEINS FROM TOMATO SEEDS

Two different methods have been applied to use tomato seed proteins for nutritional purposes: The first one consisted of grinding tomato seeds into a fine powder, often referred to as 'tomato seed meal,' which has been suggested for applications such as animal feed[25]. Further research has been executed regarding the feed quality on poultry and cattle[18,54]. The second method was the production of a protein isolate for human consumption, which has been reported to have a good nutritional value similar to that of other plant-based protein concentrates[30,55–57]. All essential amino acids are present in protein isolates derived from tomato seeds, as can be derived from Table A 1 in Appendix C (page 60).

Recently, tomato seed protein has been proven to be interracially active, and its exploitation as a stabilizer of oil-in-water emulsions has been suggested[58].

1.2.1.3 Use of pomace instead of a purified seed fraction

When developing valorization models for tomato processing residues, most authors focus themselves on either only the seed[35–37,56] or only the peel[22,59] fraction. Although this approach is useful when it comes to knowledge generation it lacks a certain realism: after all, the stream leaving processing facilities is a heterogeneous mixture of seeds, peels, parts of pulp and impurities, with variations regarding composition and humidity.

Two approaches have been described to separate the different components from each other: Kaur et al. described a 'Flotation-cum-sedimentation' system that relies on the difference in density[60]. Consequently, while the peels tend to float in water, the seeds are expected to sink to the ground. Porretta et al. refer to this system as well, and suggest a subsequent drying step in hot air tunnels[28], while Giuffrè et al. dried the separated seed fraction by exposing them to sunlight and subsequently evaporating residual humidity at 40-50 °C[61]. Drawbacks of the floating technique are the low separation efficiency as well as a relatively high water-consumption of the system. Shao et al. therefore dried the pomace and then separated the seeds with an aspirator system[36].

The cost and efficiency related disadvantages of these separation techniques illustrate why tomato residues are not being separated and valorized on a large scale, yet. While Porretta et al. allege that the development of new, tomato residue-based products would lead the tomato industry to already separate seed and peel streams throughout the process, they also express awareness that innovation is exceptionally slow to be adopted in the corresponding industry[28]. Recognizing the fact that a realistic valorization concept for tomato residues needs to be sufficiently robust to cope with fluctuating factors, Kehili et al. suggest researching the possibilities of a biorefinery cascade that processes tomato seeds and peels as one single fraction[20].

Recently, Kehili et al. showed that lycopene, supplied as a component of hexane-extracted oleoresin from tomato peels, was able to improve the oxidative stability of refined sunflower and olive oil[59]. However, high concentrations of lycopene-rich oleoresins resulted in a pro-oxidation effect in refined olive oil. This phenomenon could not be observed in the case of refined sunflower oil.

1.3 Scientific objective

This research aims to develop a novel valorization approach for tomato processing residues. The first step of the proposed biorefinery concept is a simultaneous aqueous extraction of proteins and oil bodies. Encapsulation in oil bodies is hypothesized to protect the encapsulated sensitive fatty acids against oxidation, especially ω-fatty acids, which is expected to alter the quality of the resulting product[48]. Simultaneous extraction of oil bodies and proteins might as well improve the economic feasibility of a tomato processing residues based biorefinery, and make the resulting products a cost-competitive alternative for current oil and protein sources such as soybean or rapeseed[62].

Several research questions are formulated to assess the feasibility and the efficiency of the proposed process:

[1] What is the composition of the used raw material, specifically its oil and protein content, and how does this composition influence their aqueous extraction?
[2] What is the composition and what are the physical properties of the extracted oil body-based fractions?
[3] What are the effects of co-extracted compounds on oil body properties and their stability?
[4] What is the protein profile of the oil body-based fractions? How do proteins separate between the different phases?

1.4 Motivation

The idea for this research originates from the course *New Venture Creation: From Idea to Business Plan* (MST-23406), which I followed March-May 2017 at Wageningen University. After every student had presented a business idea in a one-minute pitch, the idea behind tomato seed valorization was worked out into a business plan by a team of 5 students from different study backgrounds. This team, called initially *Tomato Seed Oil* and later renamed *Crimson gold* won the course intern competition for the best business plan out of initially 70 competitors, which resulted in a 5,000€ student start-up loan awarded by StartLife, the start-up incubator of Wageningen University.

2 BACKGROUND

2.1 TOMATO PRODUCTION FOR PROCESSING

Tomato is the second most consumed vegetable worldwide[13,63]. Since the *Food and Agricultural Organization of the United Nations* (FAO) created a FAOSTAT, a free access platform providing data regarding food production and consumption, the production volume of tomatoes increased more than 6-fold. In 2014, more than 170 Mt[a] were harvested worldwide (see Figure 2-1)[16,64].

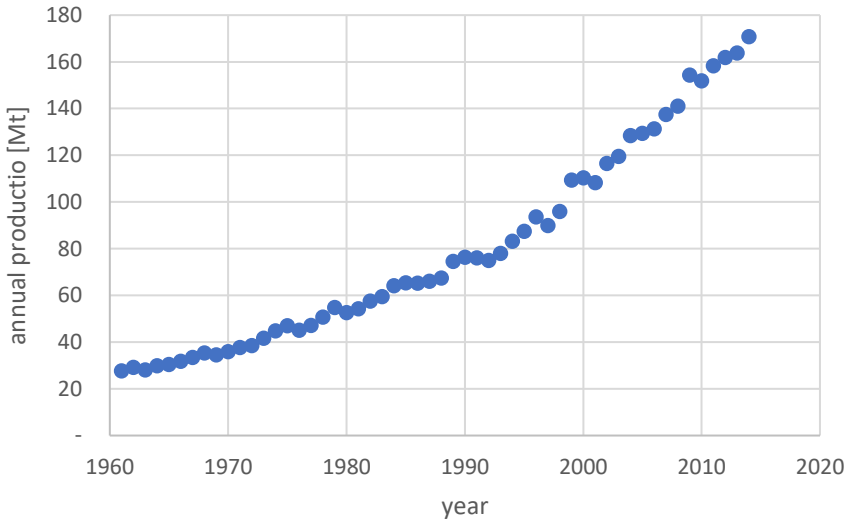

Figure 2-1. Tomato production worldwide, from 1961 until 2014 (most recent year available). Data derived from FAOSTAT.

A processing percentage can only be appreciated. Data is provided by *EUROSTAT* (www.ec.europa.eu\eurostat) and the *World Processing Tomato Council* (WPTC, www.wptc.to) butis incomplete for some regions. Nonetheless, Figure 2-2 shows a comparison between the total tomato harvest and the percentage used for processing for the ten countries producing most tomatoes.

[a] Megatonnes. 1 Mt = 10^9 kg

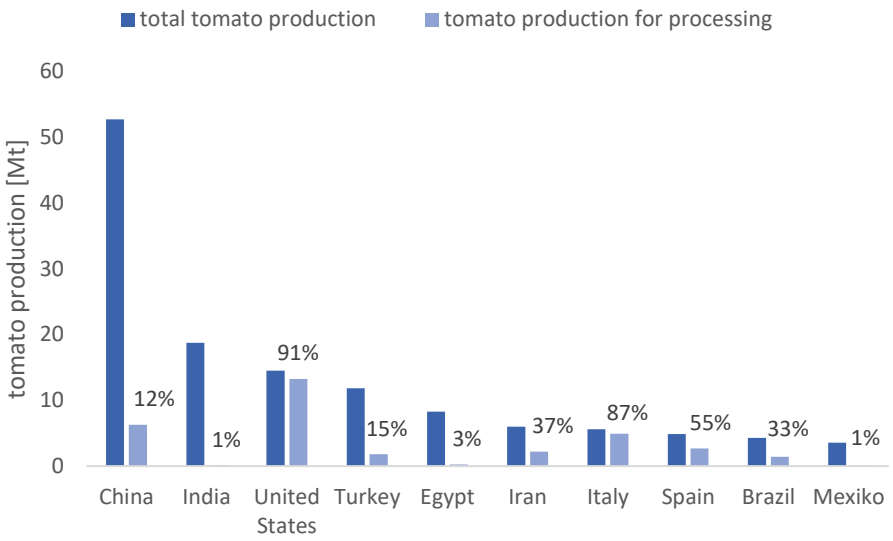

Figure 2-2. Comparison between the total production volume of tomatoes and the volume to be processed into tomato-based products. The ten most producing countries are listed. Data derives from FAOSTAT and the WPTC, for the year 2014.

Since 2005, the percentage of the annual tomato harvest to be processed altered between 20.2% and 27.6%, with an average of 23.9%[16]. Of this processing volume, roughly 4 wt.% are discarded as tomato pomace[13]. Taking into account a harvest of 170 Mt in 2014, that very same year more than 1.6 Mt of tomato pomace is estimated to have been produced.

3 Materials and Methods

3.1 Raw material

Tomato Farm S.p.A, a tomato processing company located in the province of Piedmont (Italy) kindly provided both tomato seeds and pomace for this research. Separation of the seed and peel fraction was executed by Isi Sementi S.p.A., a seed company in the province of Parma (Italy). Drying of pomace was completed in the ovens at Unifarm at Wageningen University, the Netherlands.

3.1.1 Tomato seeds

525 kg of fresh tomato pomace were collected and stored in sealable barrels for one day. Subsequently, the content of these barrels was handed over to a contractor that separated the pomace into a peel and a seed fraction, using a flotation-cum-sedimentation system similar to the one described by Kaur et al. (2005)[60]. Afterward, the purified seed fraction was dried at 40 °C for approximately four hours.

Seeds were packaged in a plastic back and stored under dark conditions and at room temperature (RT, 22 °C).

3.1.2 Tomato pomace

Fresh tomato pomace was collected and filled into barrels that were faced with an antiseptic bag. Oxygen contact was minimized, and the barrels were sealed. Shipment took place at ambient temperature and lasted three days.

The pomace was initially stored in a fridge at 4 °C for five days and was then split into two fractions: The first fraction was thinly spread to drying trays covered with aluminum foil and was dried in an industrial stove at 50 °C for 24 hours. After that, the semi-dry pomace was crumbled and mixed, to which a second drying step for another 24 hours at 50 °C followed. The dried pomace was packaged in sealable plastic bags of 3 L and stored at RT (dark conditions) until further use. The second pomace fraction was filled into sealable plastic bags of 3 L (Jumbo, the Netherlands) and stored at 4 °C for one day before being frozen at -20 °C until further use.

3.2 Chemicals

Sodium hydroxide (NaOH) was supplied by VWR International (Ohio, United States) and hydrogen chloride (HCl) was supplied by VWR Chemicals (France) with a purity of 37%.

Sodium chloride (NaCl) was supplied by Merck (Germany).

For Soxhlet extraction, PEG-grade n-hexane supplied by Actu-All Chemicals (The Netherlands) and petroleum ether from Honeywell (United States) or Acta-All Chemicals (The Netherlands) were used.

For Dumas analysis, Sigma-Aldrich (Germany) supplied both methionine for calibration (purity ≥ 98%) and cellulose for the blank.

3.3 Aqueous extraction

Aqueous extraction was first assessed on tomato seeds, using different solid:liquid ratios and extraction pHs. Upon acquisition of a good set of parameters for seeds, similar conditions were applied to dried pomace. The complete processes for seeds and pomace extraction may be derived from Figure 3-1.

3.3.1 Pre-treatment

Seeds were used entirely for starting experiments. Later, both seeds and dried pomace were ground using an IKA® M20 Universal mill (IKA-Works, Germany), after which they were sieved through a standard kitchen sieve (estimated particle size 1mm). Ground fractions were stored in sealable rice boxes (at RT), until further use.

3.3.2 Oil body and Protein Extraction procedure

The raw material was dispersed in either demineralized water or 0.5 M NaCl. The broth was stirred continuously using a head stirrer (IKA-Works, Germany). Meanwhile, the pH was adjusted using NaOH and HCl at appropriate molarities (0.5 M and 1 M for NaOH and 0.5 M and 3 M for HCl). After no less than two hours, the broth was transferred to a fridge (4 °C) overnight.

The following day, the extraction broth was blended five times using an HR2093 blender (Philips, the Netherlands). Blending intervals lasted 30 seconds at maximum speed, after which a rest phase of 15 seconds followed. Subsequently, the broth was filtered through two layers of cheesecloth, that was pressed out manually. The solid residues retained in the cheesecloth were weighed and dispersed in fresh liquid for a second extraction, which took place at the same pH as the first one and lasted at least one hour. Stirring, pH adjustment, blending, and filtering steps were executed as described before.

Centrifugation of the filtrates occurred at 10,000 g and 4 °C for 30 minutes in a Sorvall Legend XFR Centrifuge (Thermo Fisher Scientific, United States). Afterward, the newly formed cream layer (*OBC-1*) was scooped off from the top of the container and diluted 1:5 (w/v) in demineralized water or 0.5 M NaCl. The dispersed cream was agitated with a magnetic stirrer for 1 hour (level 2 on a VMS-C7 stirring plate; VWR, United States), while the pH was adjusted to the extraction pH used before. Subsequently, the dispersed cream was centrifuged for 30 minutes at 10,000 g and 4 °C. The then formed top layer (*OBC*-2) was again scooped off and weighed to determine a yield according to Equation 3.1.

$$OBC\ yield\ [\%] = \frac{weight\ OBC}{weight\ of\ raw\ material} \qquad \textbf{Equation 3.1}$$

3.3.3 Storage conditions and sample preparation for Analysis

Fresh cream fractions were used for moisture analysis. Afterward, the dried creams were transferred to Eppendorf tubes, sealed with parafilm and stored at -18 °C until oil and protein content were determined.

For particle size analysis, filtrates (*OBD-1*) were stored in 100 mL Schott bottles. The creams were diluted five times in demineralized water and stored in 15 mL Greiner tubes. All fractions were stored either at RT or 4 °C. For charge analysis, filtrate from the first extraction was diluted 100 times in MilliQ water at pH 9. Creams were first diluted five times in demineralized water and then 100 times in MilliQ water at pH 9. All samples stirred with a magnetic stirrer for at least 30 min before analysis.

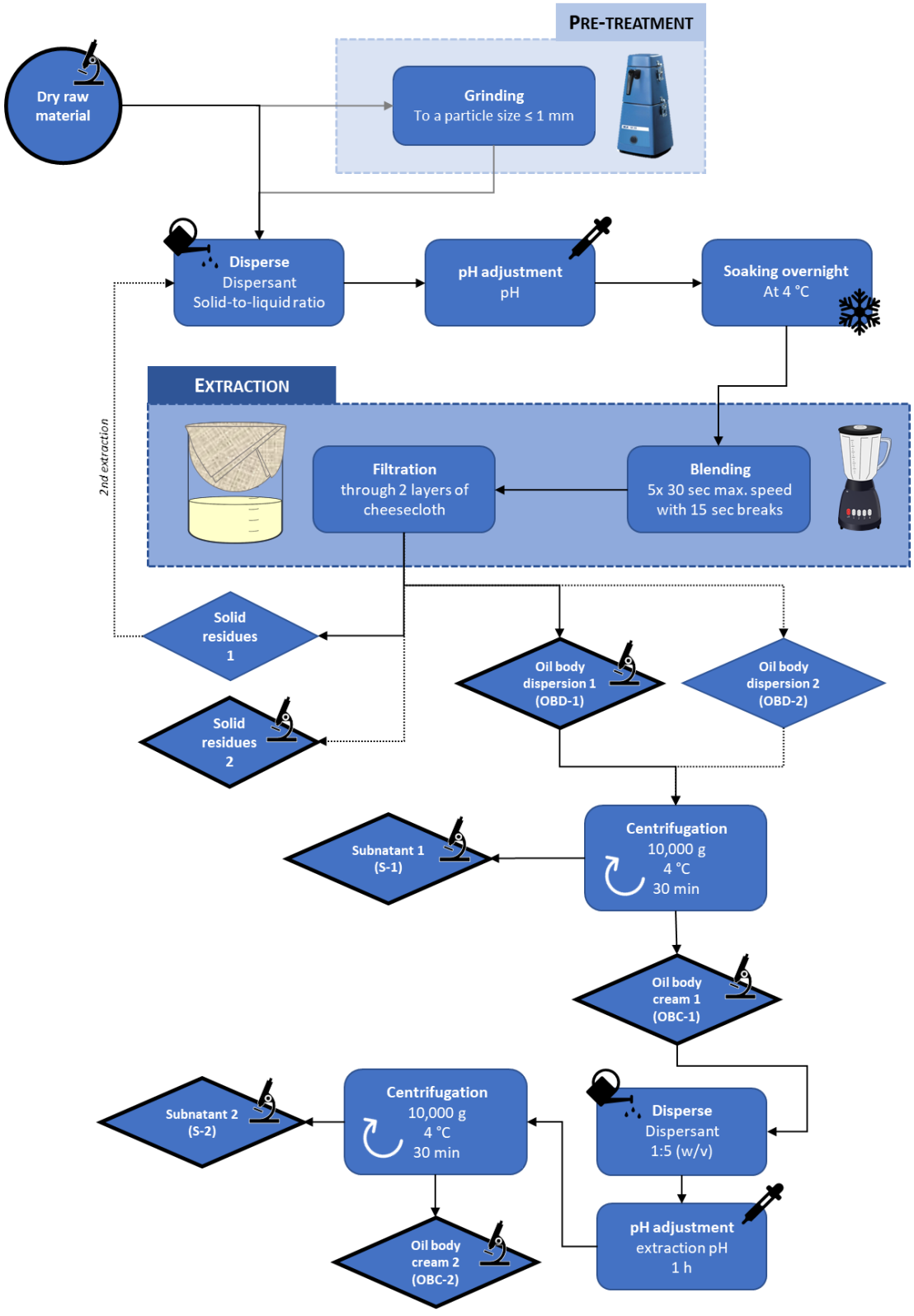

Figure 3-1. Extraction procedure as described in 3.1.1. A circle indicates the raw material, rondi stand for (interstage) products. Rectangles specify processing steps. Dotted lines indicate the second extraction step, that was ultimately not executed anymore. The grinding step was optional but was ultimately executed for better yields. The microscope symbol indicates analysis was done on this fraction.

3.4 Enzyme-assisted Aqueous Extraction (EAAE)

In an attempt to improve the effectiveness of aqueous extraction on tomato pomace, cellulose-degrading enzymes (*cellulases*) were added as suggested by Cuccolini et al.[22]. Consequently, the pre-treatment and extraction procedure presented hereafter were adapted from those authors. Their protocol was modified for this study. A graphic representation of the extraction procedure can be derived from Figure 3-2 (page 15).

3.4.1 Pre-treatment

Tomato pomace was thawed at 4 °C after it had been stored at -20 °C previously. Defrosted pomace was then mixed with demineralized water 1:4 (w/v) and blend at maximum speed in an HR2093 blender (Philips, The Netherlands), for one minute. The blend was then stirred at pH 9 for at least two hours with the aid of a head stirrer (IKA-Werke, Germany), after which the pH was decreased to 2.2. Subsequently, the blend was centrifuged in a Sorvall Legend XFR Centrifuge (Thermo Fisher Scientific, United States) for 15 minutes (2400 g, 4 °C). The supernatant was discarded.

3.4.2 Enzyme-assisted Extraction Procedure

The pellet from the centrifugation was redissolved in demineralized water at a ratio of approximately 1:2 (w/v) and pH was adjusted to 5.0. Multifect® GC extra liquid cellulase enzyme (DuPont, United States) was added at 1% weight of the wet pellet.

Enzymatic degradation occurred at 50 °C for 4 hours, during which the broth was shaken at 100 rpm in a Climo-Shaker ISF1-X (Kuhner, Switzerland). Then, the solids were filtrated off through two layers of cheesecloth, and the pH of the filtrate was adjusted to 2.2. Centrifugation at 2400 g and 4 °C for 30 minutes resulted in a bright red pellet (PC).

3.4.3 Storage conditions and sample preparation for Analysis

The fresh pellet was used for moisture analysis. Afterward, the dried fraction was transferred to Eppendorf tubes that were sealed with parafilm and were stored at -18 °C until oil and protein content were determined.

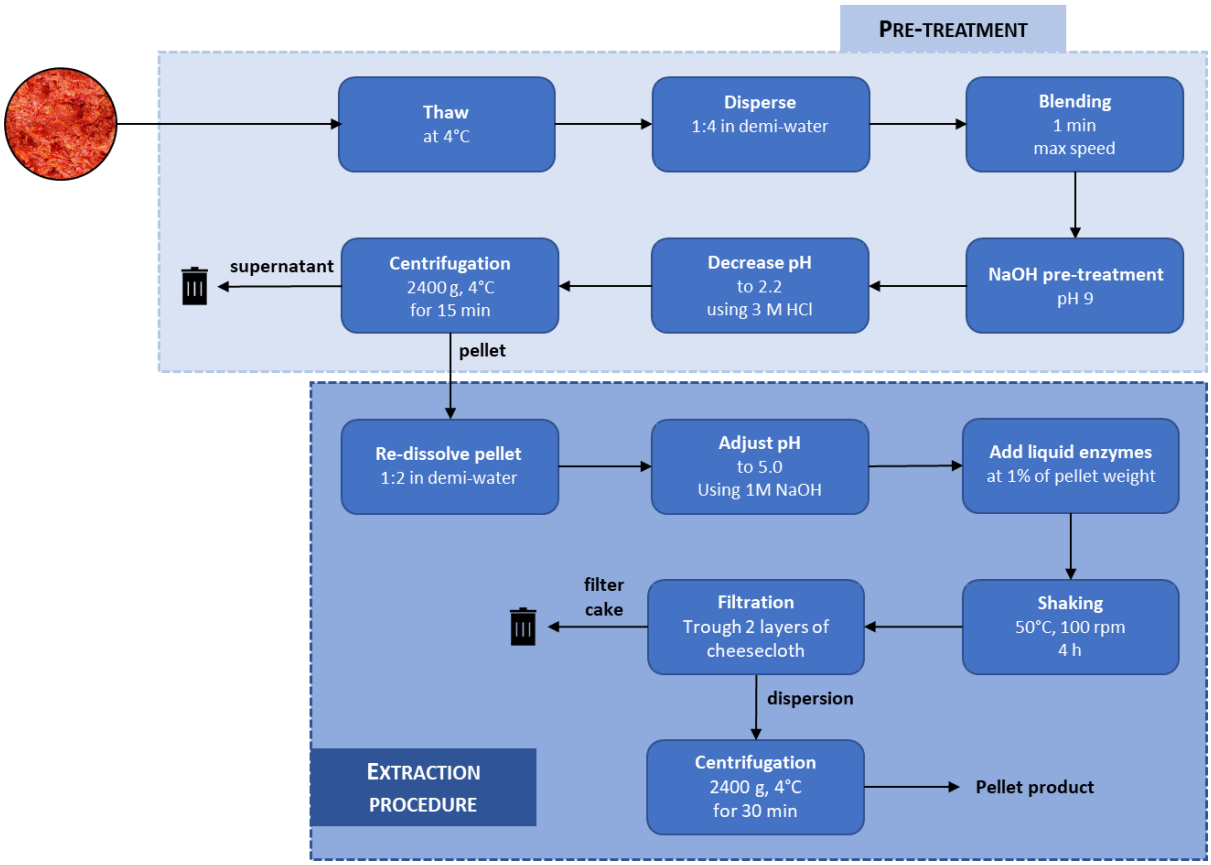

Figure 3-2. Schematic representation of the enzyme-assisted aqueous extraction procedure employed for the extraction of wet tomato pomace, based on the method described by Cuccolini et al. (2013). The waste bin depicts that the respective fraction was discarded. The final product is a bright red pellet, which was analyzed for moisture, oil and protein content.

3.5 Composition analysis

Several fractions before, during and after extraction were collected and analyzed on their content of moisture, oil, and protein.

3.5.1 Moisture

The humidity of wet and dry pomace, oil body creams, pomace creams and solid residues was determined by drying at 105°C until a stable weight was reached. Analysis was done in triplicate for each sample.

3.5.2 Oil

Soxhlet extraction was executed to determine the total oil content of the raw material, solid extraction residues, and oil body cream. Raw material and solid residues were ground as described previously.

Initially, total lipid content of the raw material was determined via hexane extraction; 30 x 80 mm thimbles (GE Healthcare, United Kingdom) were filled with either ground tomato seeds or ground tomato pomace and placed in a behrotest® Soxhlet apparatus (Behr, Germany). Both fractions were extracted with 200 mL of n-hexane for a total of 8 hours, after which the solvent was gradually drained off. After two more hours, the round bottom flasks containing oil were transferred to an oven (60 °C), were residual solvent was allowed to evaporate for 30 minutes.

Later analysis was executed using a Soxtec system HT6 (Tecator, Sweden) and 26 x 60 mm thimbles (Whatman International, England), which allowed for the determination of oil content from smaller samples. Extraction was executed using 50 mL petroleum ether per sample and consisted of 30 min of boiling in the solvent and subsequent 30 minutes of rinsing. The solvent was then collected on top of the extraction system, and the cups containing the oil were left to dry at 105 °C for 30 minutes. They were allowed to cool down in a desiccator before being weighting. Sample sizes varied between 0.5 g for oil body cream and 3 g for solid extraction residues. Analysis was done in duplicate.

Oil yields were calculated according to Equation 3.2.

$$oil\ yield\ [\%] = \frac{final\ weight\ [flask + oil] - initial\ weight[flask]}{weight\ [extractable\ raw\ material]} \qquad \textbf{Equation 3.2}$$

3.5.3 Protein

The protein content of the raw material, solid extraction residues, and oil body creams was determined using the Dumas method. Analysis was done in triplicate with the aid of a FlashEA 1112 NC Analyzer (Thermo Fisher, United States). A conversion factor of 5.7 was used to convert from nitrogen to protein content[65]. An elaborated protocol for this may be found in Appendix B.

3.6 Protein characterization

3.6.1 Pre-treatment

The subnatants of oil-body cream 1 or 2 were filtered. Subsequently, the filtered subnatants were transferred to rice boxes and frozen at -20 °C for at least 4 hours, after which the fractions were freeze-dried according to the protocol described in Appendix A.

Oil body cream samples were either used for sample preparation freshly or diluted five times in demineralized water and stored at - 18 °C until further use.

3.6.2 Gel electrophoresis

Protein samples were prepared according to the protocol given in Appendix B.

Protein size was approximated by loading 20 µL aliquots of the samples on a NuPage™ 4-12% Bis-Tris gel (Thermo Fisher, United States). 8 µL of PageRuler Plus Prestained Protein Ladder #26619 (Thermo Fisher, United States) was loaded as a size ruler. Gel Electrophoresis was executed in a Mini gel Tank filled with NuPAGE® MES SDS Running Buffer (both by Thermo Fisher, United States) and connected to a 300 V Power Source (VWR, United States), which was operated at 180 V and 90 mA for 40 minutes initially. If the smallest protein fraction from the size ruler was still far away from the lower end of the gel, electrophoresis was allowed to proceed for some more minutes, at equal flow and potential.

The gel was then freed from the plastic case, rinsed with demineralized water, placed in a rice box and covered with Coomassie Brilliant Blue. Staining took place overnight, while the gel was gently shaken at 25 rpm. Subsequently, the gel was destained by washing it with demineralized water several times. After one day of destaining at 25 rpm, the gel was stored at 4 °C until further analysis. Photographs were taken using a Huawei P9 lite or a Fujifilm X-10 camera equipped with a 27 mm lens.

3.7 Oil body characterization

3.7.1 Particle Size

50 mL of the first filtrate (*OBD-1*) where stored in 100 mL Schott bottles (50% headspace), sealed with a screw cap and stored either in a fridge (4 °C) or on a benchtop (RT). Likewise, OBC-1 and OBC-2 were diluted in demineralized water 1:5 (w/v) and stored in 15 mL Greiner tubes, either at RT or 4 °C.

The development of the particle size distribution was determined with the aid of a Mastersizer 2000 (Malvern Instruments, United Kingdom)[66]. Samples were diluted in demineralized water that had previously been adjusted to pH 9, to an oil content of approximately 0.01%. Five readings were taken per sample, and the average of these five readings was reported as the Mastersizer reading for this single sample. Samples from at least three different extractions were analyzed.

Visual proof of the particle size development data was obtained using a DMi8 microscope (Leica, Germany) with a 40x magnifying oil lens (Leica, Germany). The microscope was connected to an Orca-Flash 4.0 camera (Hamamatsu, Japan), which allowed imagining using the Leica Application Software X. Processing of the images and the addition of a scale bar were done using ImageJ (Open Source, United States).

3.7.2 Particle charge

Z-potential was measured in a Nano-ZS Zetasizer (Malvern Instruments, United Kingdom) OBD-1 and the subnatant of oil body cream 1 (S-1) were diluted 100 times in MilliQ water which had been adjusted to pH 9 with 1 M NaOH. OBC-1 and OBC-2 were first diluted in demineralized water 1:5 (w/v) and then diluted 100 times in the mentioned MilliQ water at pH 9.

Standard ζ-potential was measured in triplicate using the specifications given in Appendix A.

Autotitrations were executed either starting at pH 10 (reached via the addition of 0.5 M NaOH) and titrating down to pH 1.5 using 0.5 M and 0.1 M HCl or starting at pH 1.5 (reached via the addition of 0.5 M HCl) and the titrating up to pH 10 using 0.5 M and 0.1 M NaOH.

4 Results and Discussion

4.1 Composition of raw material

Tomato seeds had been dried to a moisture content of 6.3 wt.% directly after being separated from the peel fraction. Pomace that had been stored frozen contained 73.5±0.6 wt.% moisture, while only 2.1±0.3 wt.% water remained in the pomace fraction that had been dried.

Figure 4-1 shows the oil content of the raw material. Compared to previous investigations, the seeds in this research were relatively rich in extractable lipids. One reason for this might be that the seeds used for this study had been exposed to industrial drying, which might have resulted in lower moisture content than the sundried seeds commonly used in literature. Lower moisture content will, on the other hand, lead to a higher relative percentage of oil in the seeds.

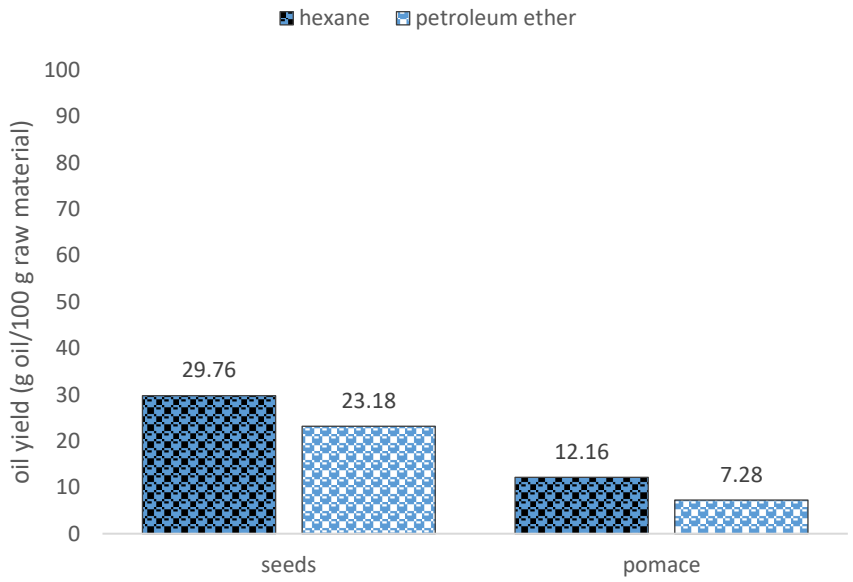

Figure 4-1. Oil content of raw materials. Note that seeds and pomace still contained 6.3% and 2.0% moisture, respectively.

Using hexane extraction on tomato seeds, Eller et al. had reported yielding 20.0 wt.% oil using accelerated solvent extraction, while Morad et al. yielded 25.1 wt.% using a modified Soxhlet apparatus[37,67]. Vági et al. had determined the hexane-extractable oil content of tomato pomace to lie between 3.39 wt.% and 15.33 wt.%, depending on the origin of the samples and their moisture content[33]. Cassinerio et al. reported an oil content of 11.29 wt.% for this fraction, which is very close to the hexane-extracted percentage obtained[18]. It should be noted that the oil content of pomace greatly depends on its specific ratio of seeds to peels, which varies depending on the tomato product it derives from.

Not all oil could be extracted within the shorter extraction time. Additionally, while petroleum ether is slightly less nonpolar than hexane; Therefore, oxidation and polymerization of oil, which might occur during storage, will influence the extractability with petroleum ether stronger than with hexane[68]. Buck and Barringer showed that while hexane and petroleum ether were equally suitable to measure

surface oil content in fresh samples, hexane extracted 25% more surface oil when the samples were one day old[68].

In line with this, seed fractions obtained in August 2017 had been analyzed on oil content using hexane in October of the same year but were only extracted with petroleum ether five months later (in April 2018). Even though the samples were not stored grounded for the full period between the two measurements, oxidation after grinding might have increased their polarity, and likewise decreased their extractability with a less nonpolar solvent.

Protein contents of raw material are presented in Figure 4-2. A broad range of protein contents, between 24.5 wt.% and 39.3 wt.%, were reported for tomato seeds[20,25]. The determined protein content of the seeds used in this study, roughly 30 wt.%, falls into this area.

Less data is available on tomato peels in general. The authors that investigated their composition so far mention values of 10.0 wt.% to 13.3 wt.%[20,25]. Dumas analysis revealed a protein content around 15.5% for the pomace fraction used in this study. As tomato seeds are known to be richer in proteins than peels, and pomace consists of varying fractions of both, the determined value is concluded to go in line with those derived from literature.

A conclusion regarding the protein content of peels cannot be drawn from these numbers yet, as knowledge on the respective proportions of seeds to peels in the pomace would be required. Those, in turns, vary depending on the season, processed variety and tomato-based product to be produced.

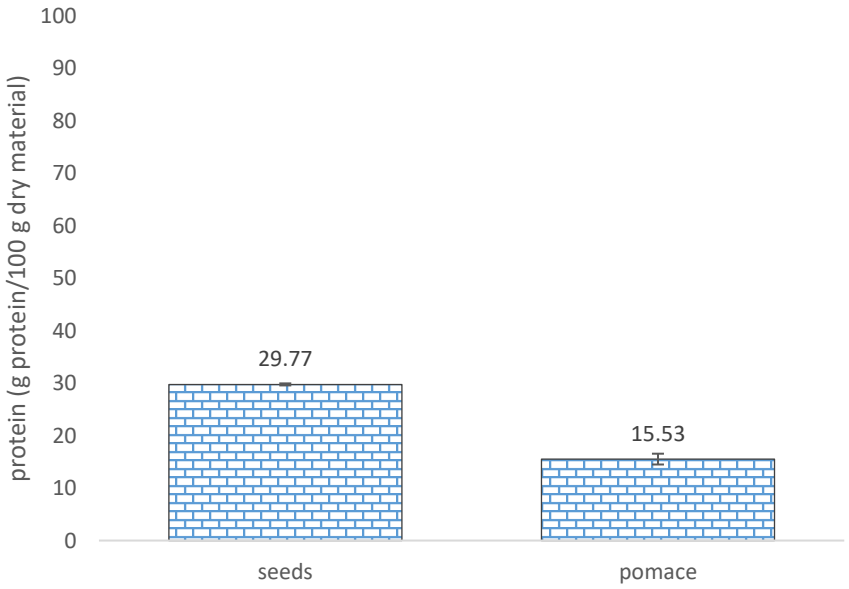

Figure 4-2. Protein content of the raw material. Protein content was corrected with the moisture content to compare with dry residues.

4.2 Parameters for Aqueous Extraction

In the early stages of this research, efforts were directed towards the acquisition of sufficient oil body cream for analysis. For this, aqueous extraction parameters were varied, which shall be described in the following paragraphs. Initially, the condition of raw material (whole seeds or seed flour) was investigated. Subsequently, pH values and solid:liquid ratios were varied to obtain enhanced quantities of cream. Finally, the influence of salt on the yield of oil body creams was assessed. The pH,

solid:liquid ratio and resulting yields of oil body cream 1 (OBC-1) and oil body cream 2 (OBC-2) of selected extractions may be derived from Table 4-1.

4.2.1 TOMATO SEEDS

For this assessment of favorable extraction conditions, tomato seeds were used, as their higher oil content compared to pomace was expected to sooner result in appropriate amounts of oil body cream.

4.2.1.1 Parameter 1 - Condition of raw material

Initially, whole seeds were used and extracted at pH 8 with an initial solid:liquid ratio of 1:4 (w/v). However, a cream layer could only be observed if the soaked seeds had been blended intensively. The yield than was around 4 g of OBC-2 from 100 g of whole seeds (4%). The extracted dispersion was brownish-opaque, which suggested the extraction of proteins at least. However, oil body extraction from whole seeds was deemed insufficient and impractical due to the low yield, which led to the usage of finely ground seeds in future experiments.

4.2.1.2 Parameter 2 – pH

The pH of the extractant has been reported to have a significant influence on the yield of oil body extractions[65]. Nikiforidis and Kiosseoglou compared extraction yields at three different pHs (3, 6 and 9) on whole Zea mays germ, its coarse flour, and its fine meal[65]. They found that aqueous extraction at pH 9 was most efficient and pH 3 led to the lowest yield. Iwanaga et al. extracted oil bodies from soybeans at pH 8.6[62].

Oil bodies repel each other when being charged, which favors extraction. When the surrounding pH is below their isoelectric point (IEP), oil bodies carry a positive charge. Contrary, they are charged negatively when the surrounding pH is higher than their IEP. Tzen et al. reported IEP values between 5.7 and 6.6 for oil bodies from various species[51]. Even though this means oil bodies are approximately neutrally charged at pH 6, Nikiforidis and Kiosseoglou detected a higher extraction yield at pH 6 than at pH 3[65]. They attributed this to the presence of extraneous proteins forming a second layer around the oil body vesicle and hypothesized that the alteration by these proteins ultimately determines the extractability of oil bodies at different surrounding pHs.

In this study, initial extractions were executed at pH 8, and a solid:liquid ratio of 1:4 (w/v) but yields of OBC-2 were low. Consequently, the extraction pH was altered to pH 9, and extraction at 1:4 (w/v) yielded 7.24% of OBC-2, which is about 80% more than when whole seeds were extracted at pH 8 (see 4.2.1.1). For a tabular representation of extractions, please see Table 4-1.

Table 4-1. Extraction yields at selected pH values and solid:liquid ratios. Ground seeds were used as a raw material. This table summarizes the numbers mentioned in section 4.2.1.2 and 4.2.1.3. Cream yields are given as a percentage of the starting quantity.

pH	Solid:liquid ratio during first extraction [w/v]	Yield OBC-1	Yield OBC-2
8	1:4	18.33%	n. a.
9	1:4	13.33%	7.24%
9	1:3.25	13.37%	6.33%
9	1:5	10.46%	8.00%
9	1:6	21.32%	10.16%
10	1:8	8.24%	4.17%
3	1:6	1.38%	n. a.

Oil bodies from various species are reported to have very similar properties, one of them being the isoelectric point[51]. Previous studies had indicated this value to lie between pH 5 and 6. Consequently, the oil bodies should be charged approximately equally strong at pH 3 (positively) as at pH 9 (negatively), repelling each other with similar forces. Assuming that no extraneous protein surrounded the oil bodies extracted from tomato seeds, an extraction at pH 3 was therefore assumed to yield quantities of oil bodies comparable to those obtained at pH 9.

However, extraction at pH 3 and solid:liquid ratio 1:6 (w/v) only yielded 1.38 % of OBC-1, which might partly be attributed to the fact that just one extraction step was executed (OBC-1 was only obtained from OBD-1). It contradicts, however, with the finding that at pH 9 at and solid:liquid ratio 1:6 (w/v), more than 88% of OBC-1 derived from OBD-1. A second extraction step could only increase the amount of OBC-1 recovered from extraction at pH 9 by 11.2%. This finding can be underpinned with data supplied by Nikiforidis and Kiosseoglou, which report that a second and third extraction only increase the total yield by few percents, independent of the pH[65].

4.2.1.3 Parameter 3 - Solid:liquid ratio

Based on the sufficient yield at pH 9 and a solid:liquid ratio of 1:4 (w/v) described in the previous section, efforts were undertaken to optimize the solid:liquid ratio for a higher yield of OBC-2.

A decrease in solid:liquid ratio was believed to improve the blending, and therefore an extraction at 1:3.25 (w/v) was executed, which was approximately the minimum ratio needed to still ensure good stirring. The final yield of OBC-2 was 6.35%, which was 0.9% less than obtained when extraction was done with a ratio of 1:4 (w/v).

Subsequently, the volume of the extractant was increased to 1:5 (w/v), which resulted in an OBC-2 yield of 8%. Further increase to 1:6 (w/v) led to an OBC-2 yield of 10.16%. Improved use of material could further enhance this yield to 10.41% and second centrifugation of the subnatant phase S-2 led to a final yield of 12.44%.

Possibly, a small solid:liquid ratio led to diffusion limitation caused by only a small difference of oil body concentrations in the solid and the liquid phase. Nonetheless, there is an optimum pH and solid:liquid ratio which should not be surpassed; extraction at pH 10 and 1:8 (w/v) only yielded 4.17% g OBC-2. Having achieved maximum yields, further extractions for analysis were always executed at pH 9 and at solid:liquid ratio 1:6 (w/v).

4.2.1.4 Parameter 4 - Salt concentration

Previous research had indicated that oil bodies extracted from soybeans were less stable to aggregation at NaCl concentrations exceeding 50 mM[62]. On the other hand, Sogi et al. refer to salt-soluble globulin compromising for 70% of the total tomato seed proteins[69]. Presence of salt is therefore hypothesized to aid the extraction of those proteins, which would subsequently facilitate the extraction of oil bodies, thereby altering the efficiency of oil body extraction.

The subnatants of the oil body cream are expected to contain all proteins that are not associated with the oil body membrane. Additionally, some of the proteins that are related to oil body membranes are expected to be found; either due to dissociation (for example when an oil body ruptured), or due to incomplete centrifugation, which would lead to some oil bodies still present in the subnatants.

Figure 4-3 (page 22) shows the size of the proteins found in the subnatants of OBC-1 and OBC-2 (named S-1 and S-2, respectively). The thickness of the bands is only a quantitative measure and shall consequently be disregarded. Band position is decisive to determine protein sizes; the similar locations across the four lanes indicate that all subnatants contain proteins of similar sizes.

Sogi et al. extensively investigated the solubility of tomato seed protein[69]. They reported three bands for alkali-extracted protein concentrate: at 19, 33 and 55 kDa when extraction was done with water, and at 20, 33 and 55 kDa when executed in 0.5M NaCl[69]. The bands visible in Figure 4-3 have sizes of approximately 20, 35 and 55 kDa. Lower molecular weight bands between 15 and 30 kDa may belong to the group of seed-type oleosins, the most abundant oil body membrane proteins[50,51,53]. The highest molecular weight band, around 55 kDa, would approximately meet the size of anther-type oleosins stabilizing the oil bodies in pollen[70]; however, those are not expected to be present in seed matrixes[53,70]. Oil body specific proteins other than oleosins express sizes around 30 kDa (caleosins) or 40 kDa (steroleosins)[53].

The band around 20 kDa is therefore assumed to belong to a seed-type oleosins, and the one around 33 kDa is supposed to depict another oil body specific protein, which might be either another oleosin or a caleosin. Regarding the band at 55 kDa, it may be assumed that it belongs to a tomato seed protein that is not classified as oil body specific but tends to attach to the oil body membrane. Globulins are the major storage proteins in seeds of spermatophytes and have also been reported to account for about 70% of the proteins found in tomato seeds[69,71]. Vicilins are a class of globulins (7S) represented by trimers composed from two different subunits with molecular weights of approximately 50 kDa and 60-70 kDa[71]. The band at 55 kDa falls into this range and is therefore believed to represent the subunit of a globulin, which attaches to the oil body membrane during extraction.

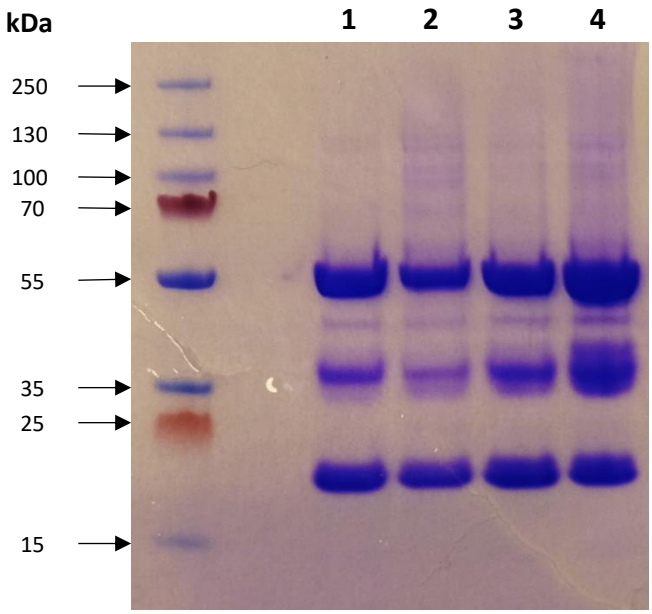

Figure 4-3. 4-12% SDS-PAGE gel showing the protein fractions freeze-dried subnatants of OBC-1 and OBC-2, when extracted either in demineralized water or in 0.5M NaCl.

[1] S-1 (Subnatant of OBC-1) from extraction in 0.5M NaCl, at pH 9 and 1:6
[2] S-2 (Subnatant of OBC-2) from extraction in 0.5M NaCl, at pH 9 and 1:6
[3] S-1 (Subnatant of OBC-1) from extraction in demineralized water, at pH 9 and 1:6
[4] S-2 (Subnatant of OBC-2) from extraction in demineralized water, at pH 9 and 1:6

All bands visible in Figure 4-3 depict similar protein sizes, no matter whether extraction was executed with 0.5 M sodium chloride or with plain demineralized water. Additionally, there was little difference

in oil body cream yield noted, with the extraction performed with demineralized water yielding slightly more OBC-2 (data not shown). Therefore, further extractions were executed without additional salt.

4.2.1.5 Final extraction conditions

This investigation aimed at finding proper parameters regarding raw material condition, pH, solid:liquid ratio and salt concentration to obtain sufficient amounts of OBC-1 and OBC-2 for further analysis. Aqueous extraction of grounded tomato seeds at pH 9 and a solid:liquid ratio of 1:6 in demineralized water was shown to yield good amounts of OBC-2. At these conditions, the second extraction yielding in OBD-2 hardly improved the yield of OBC-1 anymore (88.8% of OBC-1 derived from OBD-1). Therefore, only one extraction yielding in OBD-1 was executed for particle and composition analysis.

4.2.1.6 Extraction Efficiency

The extraction efficiency of oil and proteins from tomato seeds can be assessed by comparing the compositions of raw material and solid residues, as well as the mass difference of dry matter. Figure 4-4 shows the oil content of solid residues.

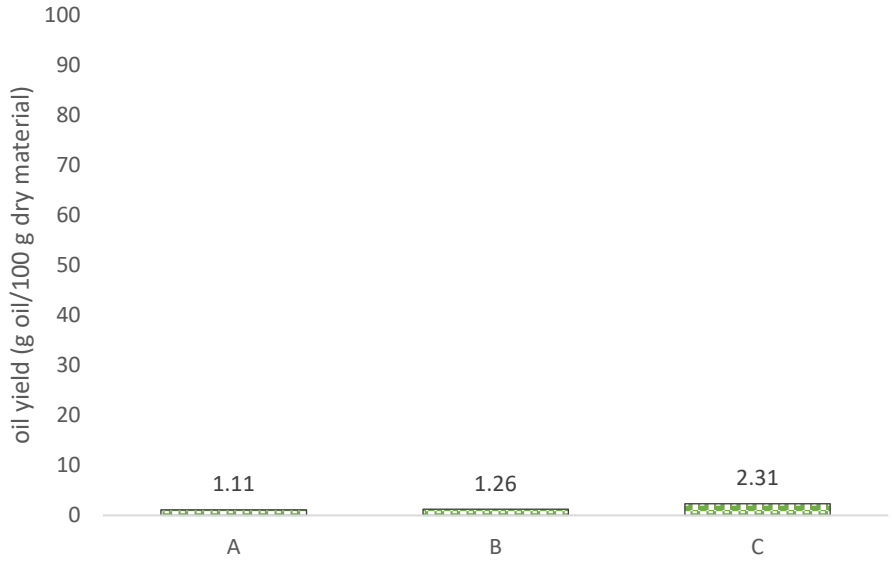

Figure 4-4. Average oil content of solid residues from 3 different extractions.

While the original seeds were relatively rich in oil (up to 31.64 wt.% on a dry weight basis when extracted with hexane), close to no oil could be detected in the solid residues anymore. The extraction efficiency of oil from the raw material is therefore expected to be high under the chosen conditions. Table 4-2 uncovers the mass of oil still present in solid residues, and the resulting extraction efficiency for oil.

Table 4-2. Oil extraction efficiency during aqueous extraction in demineralized water, at pH 9 and a solid:liquid ratio of 1:6

Replicate	**Oil [g]**	**Oil extracted with aqueous extraction**
A	0.42	98.57%
B	0.46	98.45%
C	0.88	97.06%

It should be emphasized that high oil extraction efficiencies from the raw material do not automatically translate to a good yield of oil body cream, and therefore a good yield on the whole process. It is possible that part of the extracted oil bodies is lost during operation, or that oil bodies are not separated appropriately from dispersing phases.

While the oil content of solid residues was very small, this cannot be stated for the protein content. Figure 4-5 shows that the weight percentage of proteins in the solid residues is only insignificantly smaller than that of the raw material (29.77 wt.% on average, expressed in Figure 4-2). All residues collected and analyzed for this graphic derived from extractions of grounded seeds in demineralized water, at pH 9 and solid:liquid ratio 1:6. The small range that all values fall into suggests a high reproducibility. Similar conditions are applied for aqueous extraction of protein extracts from plant matrixes and have also been applied to tomato seeds in the past, yielding decent amounts of protein[30,56].

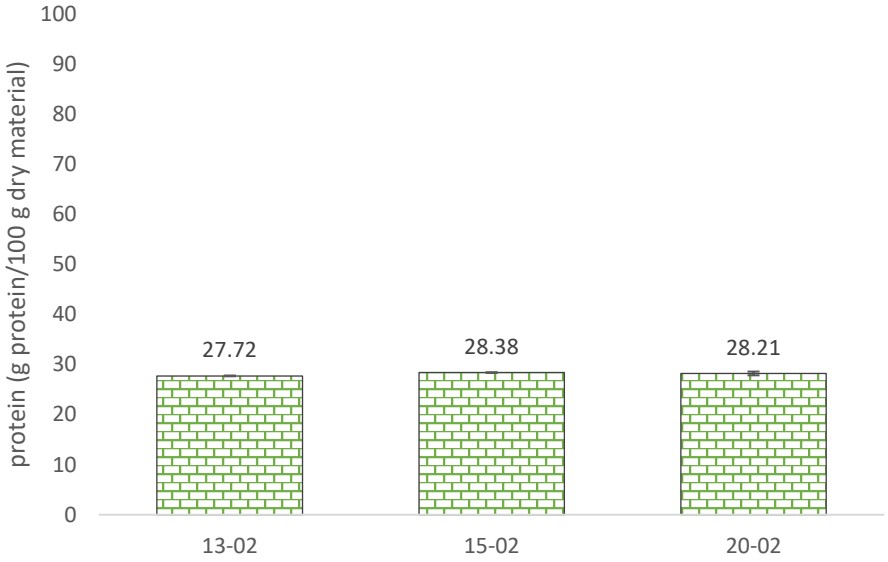

Figure 4-5. Protein content of solid residues from 3 different extractions. Error bars indicate the analytical error between three different measurements on the same sample.

Comparing the protein mass (in g) remaining in the residues with the one of raw material reveals that protein was extracted with an efficiency of 62.38±0.48%. Therefore, the values from Figure 4-5 may be explained by an overall decrease in mass.

Table 4-3. Protein extraction efficiency during aqueous extraction in demineralized water, at pH 9 and a solid:liquid ratio of 1:6

Replicate	**Protein [g]**	**Protein extracted with aqueous extraction**
A	10.58	62.20%
B	10.35	63.04%
C	10.67	61.90%

4.2.2 TOMATO POMACE

The same procedure as for seeds was tested using dry tomato pomace as a raw material. Pomace was grounded into a fine powder to maximize extraction efficiency, which was deemed more important as it contained less oil than purified seeds and oil body cream yield needed to be sufficient for analysis. Subsequently, the same protocol as for tomato seeds was employed for extraction.

Issues arose first and foremost regarding the volumes of water needed. While seeds required only three times their weight in volume to receive a consistency that could be stirred easily, the dried pomace was very sticky even when being mixed with six-time its mass in demineralized water. By the end of the pH adjustment, it had been dispersed in demineralized water more than ten times (1:11.1 w/v).

Residues after the first cheesecloth filtration were relatively heavy (approximately 380 g; seed cakes weighed around 200 g after the first filtration). This gain in weight suggests that the peels, rather than being extracted, had soaked up much water.

The first cream to be obtained from this extraction was the pomace equivalent of OBC-1, which can be seen in Figure 4-6. However, this fraction was small in quantity; only 1.63 g could be obtained from 100 g of pomace, which was too little for analysis.

One reason for the low yield might be that OBD-1 and OBD-2 had been combined before centrifugation. For seed extractions, it had been observed that the majority of cream could be yielded from OBD-1. Combination of OBD-1 and OBD-2 then results in a lower overall volume percentage of oil bodies, which in turns facilitates dissolving of the produced cream in the underlying subnatant. Mentioned dissolving phenomenon could be observed for the case of the produced oil body cream from pomace.

Figure 4-6. The equivalent of OBC-1 obtained from aqueous extraction of pomace at pH 9. Flakes were unstable and dissolved quickly in the underlying subnatants.

The presence of lignocellulosic material may have additionally hampered extraction efficiency. According to Toscano et al. (2015), the hemicellulose, cellulose and lignin fractions in tomato peels account for 4.8%, 22.5% and 46.9% of the dry mass, respectively[31]. These polysaccharides and polymers provide structure and stability to the peels, but at the same time bedevil extraction of components out of the tight network they spin. Based on those findings, enzyme-assisted aqueous extraction of tomato pomace shall be described in the following section.

4.3 Enzyme-Assisted Aqueous Extraction of pomace

The low oil body cream yield from pomace was partly attributed to the presence of polysaccharides in the peels, which had previously been reported to be rich in lignocellulosic material[20,31]. However, Cuccolini et al. (2013) had recently developed a method to extract lycopene-rich chromoplasts from tomato peels[22]. As seed extraction had already been shown to be possible and the issue in pomace extraction seemed to be related to the peel fraction, the protocol developed by Cuccolini et al. was adjusted for this study.

Figure 4-8. The first pomace pellet to be resuspended and enzymatically hydrolyzed.

The procedure included an alkali pre-treatment, followed by pH reduction and centrifugation, after which the supernatant was discarded. The pellet (visible in Figure 4-8) was re-suspended, and the pH was adjusted before enzymatic hydrolysis. Afterward, solids were filtrated off, and the chromoplasts containing the lycopene were precipitated at the isoelectric point of their surrounding proteins. The pellet from the subsequent centrifugation then contained a bright red pellet visible in Figure 4-7, which was called *'pomace cream' (PC)* and analyzed together with the oil body creams from seeds.

Figure 4-7. The red pellet produced by enzyme-assisted aqueous extraction of tomato pomace. Left: centrifugation bottle directly after centrifugation. The yellowish supernatant was discarded. Middle: Pellet from above, without supernatant. Right: pellet once scooped out of the centrifugation bottles.

4.3.1 Extraction efficiency

Contrary to the seed extraction (where raw material, solid residues, and oil body cream were analyzed), the extraction efficiency of the pomace can only be estimated based on the oil content of the raw material and the product that has been obtained. The solid residues of EAAE were not analyzed. An extraction starting with unfrozen pomace yielded 11.39% of pomace cream. The overall yield shall be discussed later, after the component analysis of the creams in section 4.4.2.

4.4 Oil body cream characterization

Alkaline aqueous extraction of ground tomato seeds resulted in a dense oil body cream. Both this cream (OBC-1) and its washed variant (OBC-2) were analyzed for moisture, oil and protein content, Pomace cream (PC) from enzyme-assisted alkaline extraction of thawed tomato pomacen was analyzed on the same compounds.

Alkaline extraction of dried pomace only resulted in few oil body cream flakes when executed under the same conditions that had been proven successful for the seed extraction. These flakes were drifting on the surface of the subnatants after centrifugation, in which they dissolved rapidly. Due to the difficulties to separate the two phases properly and obtain an amount of pomace oil body cream sufficient for analysis, alkaline aqueous extraction of dried tomato pomace was not investigated further.

4.4.1 Oil body cream composition

Figure 4-9 shows the moisture content of OBC-1, OBC-2, and PC. Contrary to the oil body creams, PC contained large percentages water. Possibly, this might be related to hygroscopic characteristics of the peels, which would also explain the large volumes of dispersant that were needed for the aqueous extraction of dry tomato pomace.

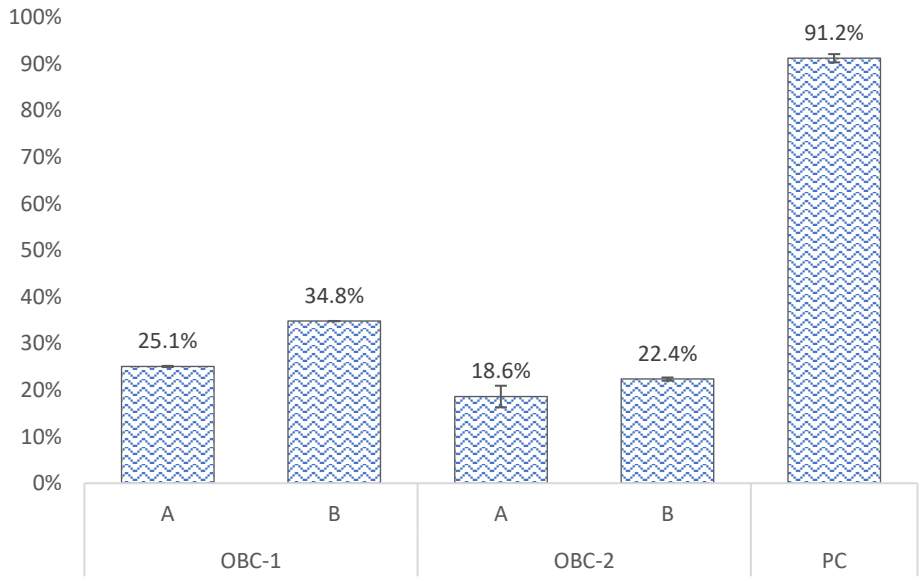

Figure 4-9. Moisture content of oil body cream 1 (OBC-1), oil body cream 2 (OBC-2) and the pellet product from enzyme-assisted aqueous extraction (PC). Error bars give the standard deviation from analysis.

The two samples of OBC-1 still differ significantly in moisture content (9.7%), while the difference in water content between the two replicates of OBC-2 is only small (3.8%). This could be explained by a difference in density between the two cream fractions, and the method used for recovery. OBC-1 was still separated from large liquid volumes. Contrary to this, OBC-2 was only diluted five times in demineralized water, after which centrifugation often took place in 50 mL Greiner tubes, being tighter than the centrifuge bottles used for OBC-1. Both factors might have benefitted the formation of a denser cream that could easily be separated from underlying liquid phases, thereby avoiding unnecessary spooning of fluid.

Figure 4-10 shows the average oil content of OBC-1, OBC-2, and PC as determined on dry samples. Strikingly, these results suggest that OBC-1, while being the first cream to be obtained, is richer in extractable lipids than its product, OBC-2. The contrary would have been expected; the washing step on OBC-1 was executed to remove foreign proteins attached to the oil bodies, which should have resulted in purified oil bodies in OBC-2. This phenomenon will be elaborated on further in a later subchapter (4.4.4).

PC expressed the lowest oil yield measured for a cream product, which was already hypothesized after drying; while OBC-1 and OBC-2 were of sticky, honey-like consistency, PC consisted of dark flakes without any visible oil.

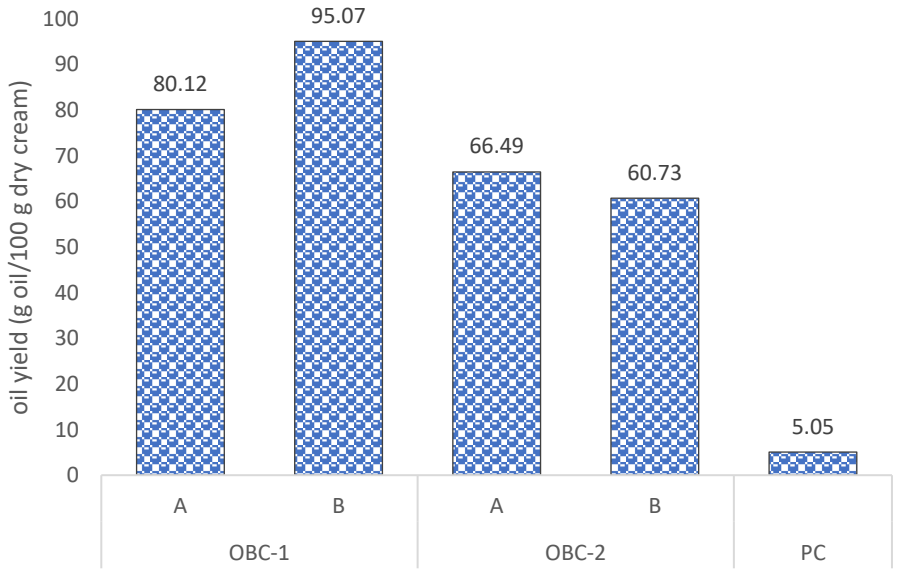

Figure 4-10. Petroleum ether extractable lipids as a dry weight percentage of OBC-1, OBC-2, and PC.

Figure 4-11 shows the protein content of dry OBC-1, OBC-2, and PC. The protein content of the oil body creams from seed extractions was very low; OBC-1 contained about 1% protein, while less than 0.5% of protein could be detected in OBC-2. PC was rich in proteins, which might be related to the fact that for its acquisition, a pellet has been used instead of a floating cream layer. This pellet on the bottom of the centrifuge tube also formed when seeds were extracted. However, it was typically not collected for analysis. It may be hypothesized that it consisted of proteins mainly, therefore resembling the composition of PC.

Tzen et al. (1993) reported protein contents from various species to contain between 0.59% (sesame) and 3.46% (rapeseed) of protein on a dry weight basis[51]. While the protein concentrations presented here are rather low, they do fall into this range.

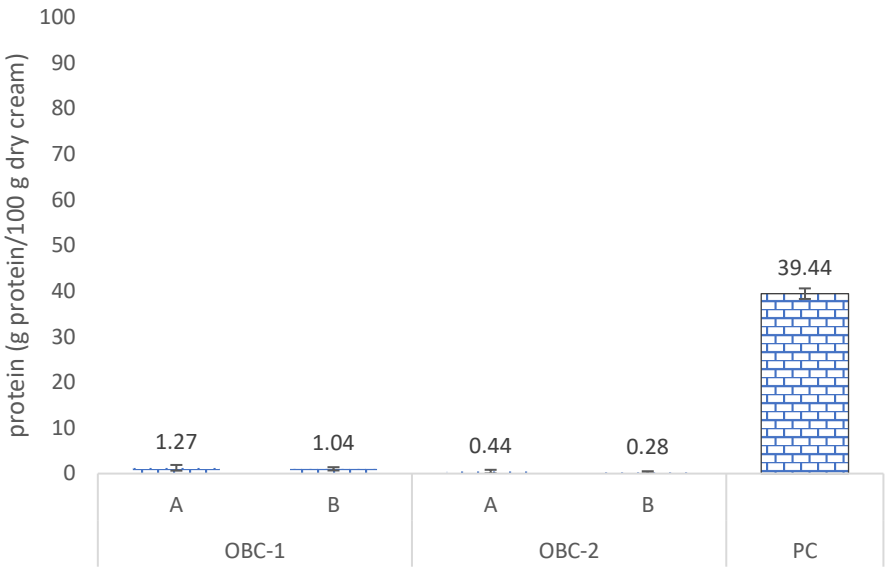

Figure 4-11. Protein content of OBC-1, OBC-2, and PC. Error bars indicate standard deviations from analysis.

4.4.2 Oil body specific proteins

Freshly prepared OBC-1 and OBC-2 were and loaded on a 4-12% Bis-Tris gel, which is depicted in Figure 4-12.

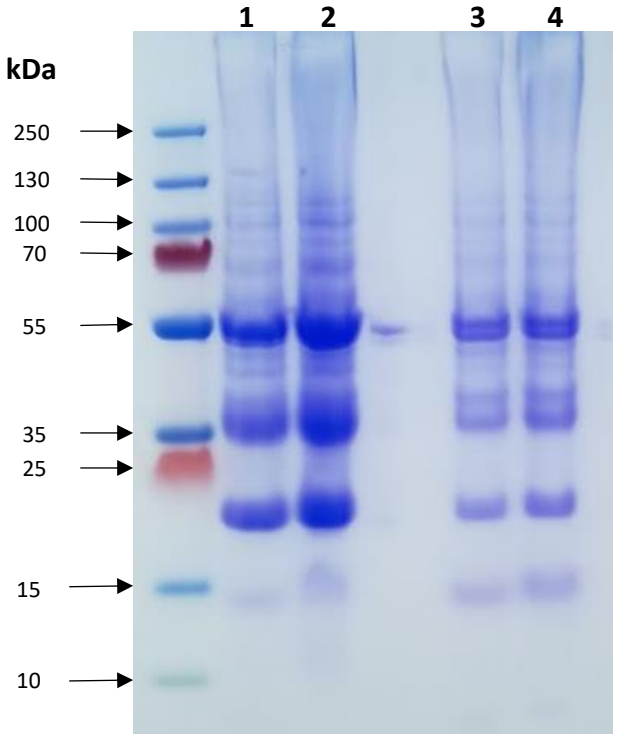

Figure 4-12. 4-12% SDS-PAGE gel showing the protein fractions from fresh OBC-1 and OBC-2 (extraction with demineralized water at ratio 1:6, at pH 9)

[1] & [2] OBC-1
[3] & [4] OBC-2

The bands resemble those encountered in the subnatants of the creams (see Figure 4-3 on page 22). However, the band at 15 kDa length does not appear in the supernatants, which might also be due to the low concentration (it is the palest of the 4-5 visible fractions). Based on its molecular weight, it may represent a type of oleosin[53,72]. The bands do not change regarding protein sizes from between OBC-1 and OBC-2. Therefore, even if the total protein content was decreased after the washing step at pH 9 (see Figure 4-11), it is questionable whether all exogenous proteins that were present in OBC-1 were removed. Washing at a pH that would result in acid precipitation of the extraneous proteins might more efficiently purify the oil bodies[48].

4.4.3 Overall yield and fat/protein ratios

Analysis on oil body fractions was done on dry samples. Composition was therefore calculated back to wet weight, which resulted in the values given in Table 4-4.

Table 4-4. Moisture, oil and protein content of OBC-1 and OBC-2.

	Moisture	Crude fat	Total protein	Fat/protein ratio
OBC-1	29.93±4.87%	61.01±0.97%	0.82±0.14%	76.94 : 1
OBC-2	20.49±1.87%	50.63±3.48%	0.29±0.07%	185.16 : 1

To assess the overall feasibility of a process, the yield of extraction is essential, which requires an accounting of masses. Many extractions have been executed for grounded seeds at pH 9 in 1:6 (w/v) demineralized water. The weight of both OBC-1 and OBC-2 differed slightly per extraction. Weights from three different extractions executed under the same conditions may be withdrawn from Table 4-5.

Table 4-5. Mass yields of OBD-1, OBC-1 and OBC-2 from three different extractions at pH 9 and 1:6 (w/v) in demineralized water, done for analysis. Samples of OBD-1 and OBC-1 were withdrawn for analysis, which was corrected for.

Extraction	**OBD-1 [mL]**	**Yield OBC-1 [g]**	**Yield OBC-2 [g]**
A	430	15.61	8.44
B	415	14.94	10.37
C	415	13.67	11.71
AVERAGE		**14.74±0.81**	**10.17±1.34**

The average of those values was assumed to be a good estimate of the yield to be obtained and was used to obtain the yields presented in Table 4-6.

Table 4-6. Oil and protein yield in OBC-1 and OBC-2 from the extraction of grounded seeds in 1:6 (w/v) demineralized water and pH 9.

	Crude fat	Total protein
OBC-1	30.22±0.48%	0.43±0.07%
OBC-2	17.31±1.19%	0.10±0.03%

Iwanaga et al. report a yield of 36% for their final cream and suspect that most of oil was retained in the filter cake. However, analysis of the dry residues in this study revealed they nearly do not contain any lipids anymore. Oil and protein may have been lost due to insufficient separation techniques.

Dry pomace contained 7-12% oil and 15% protein approximately (see Figure 4-1 and Figure 4-2), and this percentage is even lower when wet pomace is used as a starting material. Moisture content analysis revealed that wet pomace consisted of 73.5±0.6% water, which brings down both contents to about a quarter of their dry weight percentage. Table 4-7 compares moisture, oil and protein content of wet tomato pomace and the pomace cream that was extracted from it.

Table 4-7. Moisture, oil and protein content of wet pomace and the product extracted from it.

	Moisture	Crude fat	Total protein	Fat/protein ratio
Pomace	73.51±0.58%	1.93 - 3.22%[a]	4.03±0.27%	0.80 : 1
PC	91.22±0.89%	0.44%	3.46%	0.13 : 1

As mentioned before, extraction of 200 g wet pomace yielded 22.78 g PC. As suggested by the low oil percentage in Table 4-7, the yield on oil is very low as can be seen in Table 4-8. The yield on proteins is somewhat higher; the clear majority of the pomace cream, however, is moisture. It is believed that polysaccharides from tomato peels are highly hygroscopic, soaking up dispersant and therefore even enriching a pellet in water. This issue has not been investigated further.

Table 4-8. Oil and protein yield from EAAE of pomace

	Crude fat	Total protein
EAAE of pomace	1.57 – 2.62%	9.78%

In the raw material, the oil/ratio is approximately equal to one. During the purification of oil bodies, the oil content increases and consequently the ratio shifts significantly. This trend is even stronger in OBC-2: while the overall oil content was decreased by about 42%, more than three quarters of the protein were removed. The pomace cream shows an atypical ratio, as the oil content is smaller than the protein content. However, comparison between PC and oil body creams is questionable due to the different extraction methods employed.

4.4.4 Oil and protein loss during extraction

The results presented in the previous sections show that the oil yield in the form of oil bodies is not optimal yet. Previous studies were not directed towards yield optimization of the extraction process and explained low yields with oil to be retained in the filter cake[62]. However, analysis of the filter cake fraction from this study showed it contains less than 2% of the original amount of oil. In an effort to analyze loss and optimize future operations, mass balances were set-up for oil and protein.

Figure 4-13 shows graphical representation of these mass balances. Two points of loss were analyzed; the upper point being the difference of oil respectively protein content of the raw material and the sum of the oil respectively protein content in the solid residues and OBC-1. The lower point of loss was the difference between oil respectively protein content of OBC-1 and OBC-2.

[a] Depending on extractant used

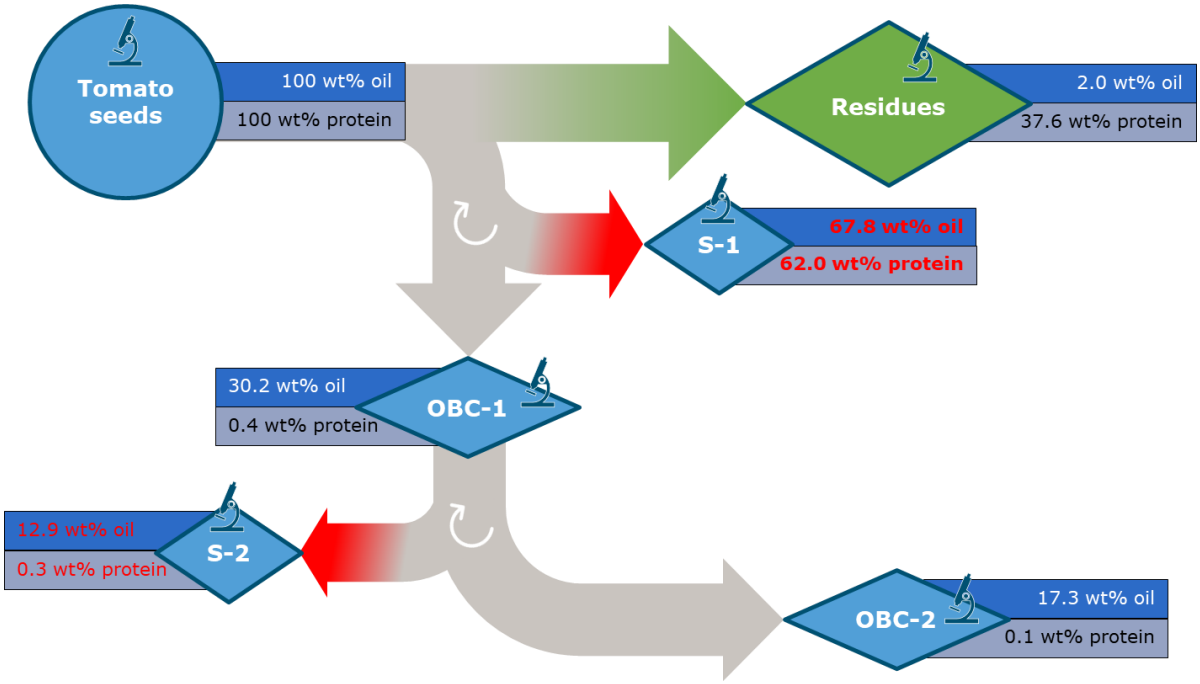

Figure 4-13. Distribution of oil and protein over the different samples that were analyzed. Arrows ending in red indicate loss of components. Weight percentages refer to the amount present initially.

In terms of mass, most loss occurs in the upper point, which might be because this phase includes many more operations; loss of oil and protein might occur due to the loss of raw material, e.g. when a beaker glass is changed, or part of the raw material is retained in the cheesecloth used for filtration. Protein loss is additionally expected during centrifugation, when the protein-rich subnatant of OBC-1 is discarded. Loss of oil might also occur during centrifugation, as it is possible that not all oil bodies result in the upper cream layer.

4.5 Oil body analysis

To assess possible applications of oil bodies and their suspensions, knowledge about their specific properties and stability is crucial[62].

4.5.1 Size distribution

Particle size distribution over time is an important parameter to assess the stability of oil bodies. Oil bodies vary in size depending on the type of matrix they have been extracted from but are generally said to be 0.5-2 µm in diameter[51,53,73]. They are stabilized by a monolayer of phospholipids into which oil body membrane proteins are embedded[53]. These proteins have been suggested to act as regulators for the size of the oil bodies[50,51,53]. Apart from the membrane proteins, extrinsic protein has been proposed to attach to the oil body surface and form an additional layer around the oil body interface[48,73]. This second layer is believed to both improve the oxidative stability of the enclosed fatty acids and stabilize the single oil body units.

Both types of proteins need to be considered regarding particle size development. Proteins are charged positively when the surrounding pH is below their individual IEP and negatively when the surrounding pH is above this value. Similar charges lead to repulsion of compounds and complexes from each other, which prevents aggregation and coalescence. Contrary, when the surrounding pH matches the IEP and the proteins are charged neutrally, aggregation of oil body complexes is favored.

Depending on the presence of extrinsic protein on the oil body surface, either those or the oil body membrane specific proteins determine if and to which degree aggregation occurs.

In OBD-1, extrinsic proteins and oil bodies were stored together in a dispersion and interaction of them is very likely. OBC-1 is a cream phase, but extraneous proteins are believed to still form a second layer around the oil bodies. Depending on the surrounding pH and the IEP of those proteins, this attachment might either stabilize or destabilize the oil bodies. In any case, the effect through extrinsic protein is thought to be less for OBC-2, as the executed washing step has previously been shown to decrease overall protein content by roughly three quarters.

4.5.1.1 Size distribution over time in OBD-1

Figure 4-14 (page 35) shows the volume occupied by particles with a given diameter in OBD-1 over a time frame of five days. After this moment, microbial growth had been reported for other types of emulsions. Indeed, microscopic analysis revealed the presence of microbes after seven days of storage, at least.

At RT, a shift in size distribution can already be observed after one day of storage. From this moment onwards, the particle size distribution remains approximately the same. It appears the curve of samples after three days is lightly shifted to the right still, which could be explained by two different reasons: one the one hand side, the shift is only small, and differences of the order shown here could be attributed to differences between different samples from different extractions, and measurement errors. A second option might be that microbial growth already influences the size distribution after five days of storage, which therefore appears to have slightly smaller particles than it would have if no microbial growth took place.

In fact, only one sample had both been measured after three days and five days of storage; The mean particle size in this sample is slightly decreasing in the given time frame, from 13 µm to 11 µm. Possibly, particles become smaller through microbial activity or enzymatic degradation. When stored at 4 °C, the size distribution stays a lot more stable than at RT. Only after five days, particles with sizes >10 µm appear, but their volumetric percentage of the whole dispersion is low.

Figure 4-15 (page 36) shows Microscopic pictures of OBD-1 directly after extraction, and after one day, three days and five days of storage; either at RT or at 4 °C. Size distribution analysis via light scattering had suggested the earlier formation of larger complexes at RT, and this trend can be confirmed from the pictures: at RT, clusters and chains of oil body aggregates can be observed already after one day of storage. While to a limited extent this phenomenon also occurs at 4 °C, it is notably less, and the overall oil body distribution appears to be evener.

4.5.1.2 Size distribution over time in OBC-1

Compared to OBD-1, the particle sizes of OBC-1 stored at RT stretch out more evenly, but particle sizes keep increasing up to the fifth day. Microscopic analysis revealed the formation of large units, which had partly opened up and released oil (see Figure 4-17 on page 38). Iwanaga et al. (2007) reported the formation of large, irregularly floc-shaped particles at pH values close to the isoelectric point of the analyzed soy bean oil bodies[62]. They provide decreased electrostatic repulsion between the oil bodies, which might have led to aggregation, as a possible reason for this phenomenon.

However, the storage pH was equal for the dilutions stored at RT and 4 °C, with the fractions stored chilly expressing much less formation of large particles. Figure 4-16 shows that at 4 °C the mean particle diameter shifts only shifts after five days of storage, and the overall size distribution becomes larger. The difference between the samples stored at RT and 4 °C suggests temperature-dependent degradation, which might be caused by enzymes. For the case of soybeans, association of

phospholipases with the oil bodies has been suggested, which would be capable of rupturing part of the oil body surface[62].

4.5.1.3 Size distribution over time in OBC-2

Compared to OBC-1, the second cream remains much more constant regarding particle size distribution, even at RT. While the initial mean particle size is increased compared to OBC-1, size gain upon storage is slowed down. Assuming enzymes caused the effects in OBC-1, this suggests that at least part of them must have been removed during the washing step at pH 9. Formation of large particles still occurs, as can be observed from the pictures represented in Figure 4-19. Especially after five days of storage at RT, large oil bodies with a diameter around 50 µm had been formed. However, no ruptured or open oil bodies could be observed for OBC-2.

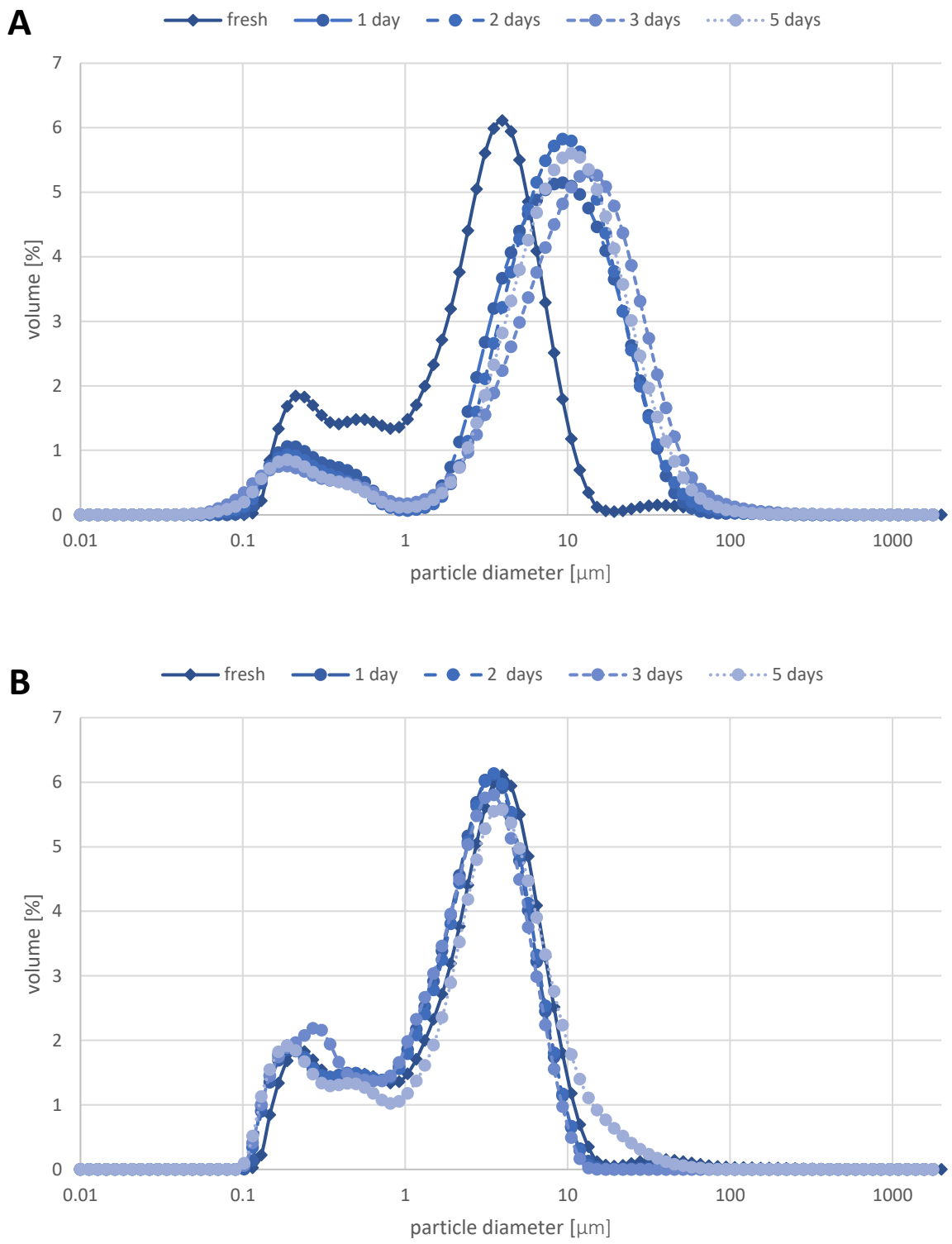

Figure 4-14. Particle size development of OBD-1. A – during storage at RT; B - during storage in a refrigerator (4 °C). Points indicated with a circle (●) have been determined as an average from measurements in triplicate, while points indicated with a diamond derive from measurements executed in quintuplicate.

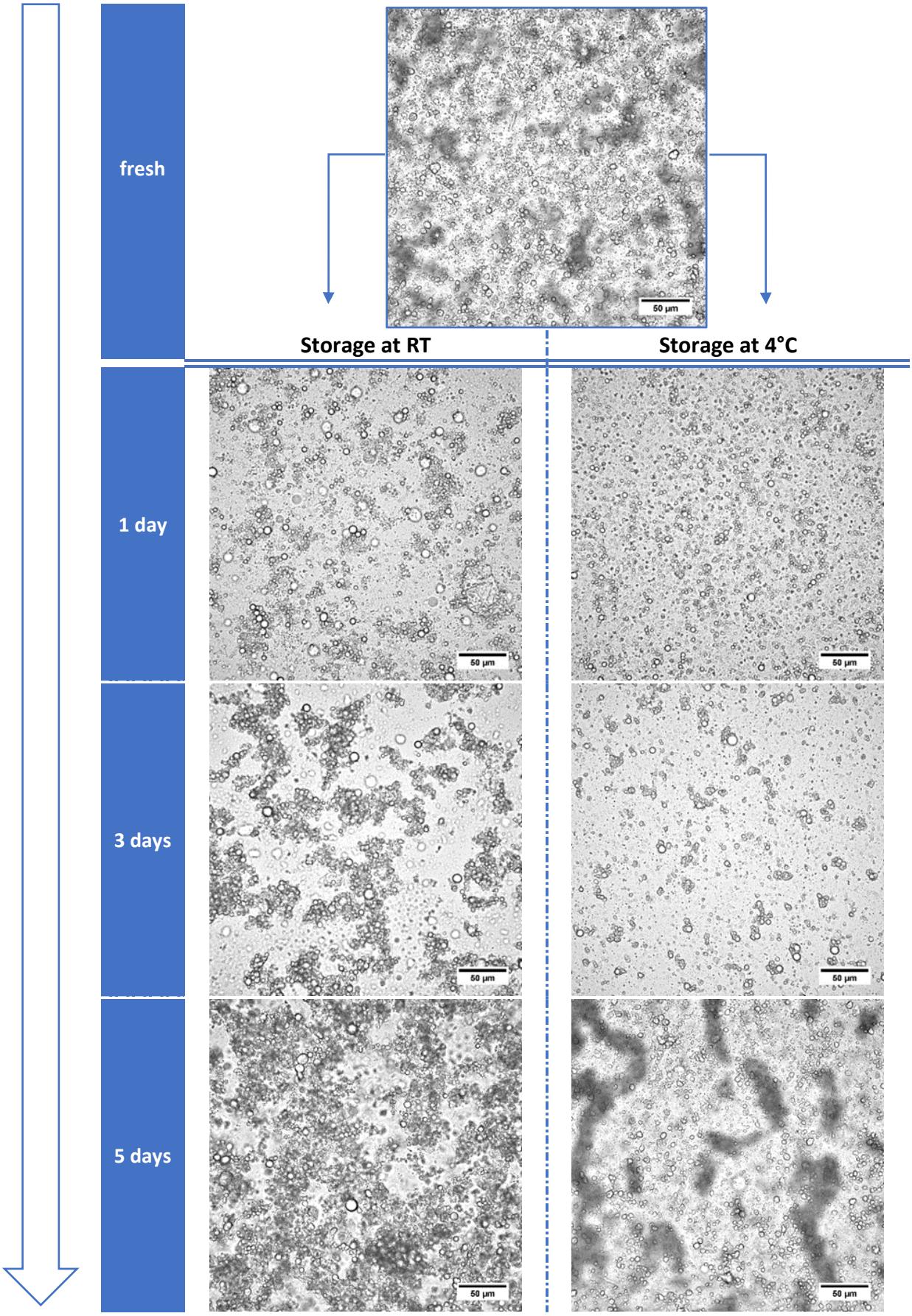

Figure 4-15. Microscope pictures of OBD-1 when stored at RT or 4°C.

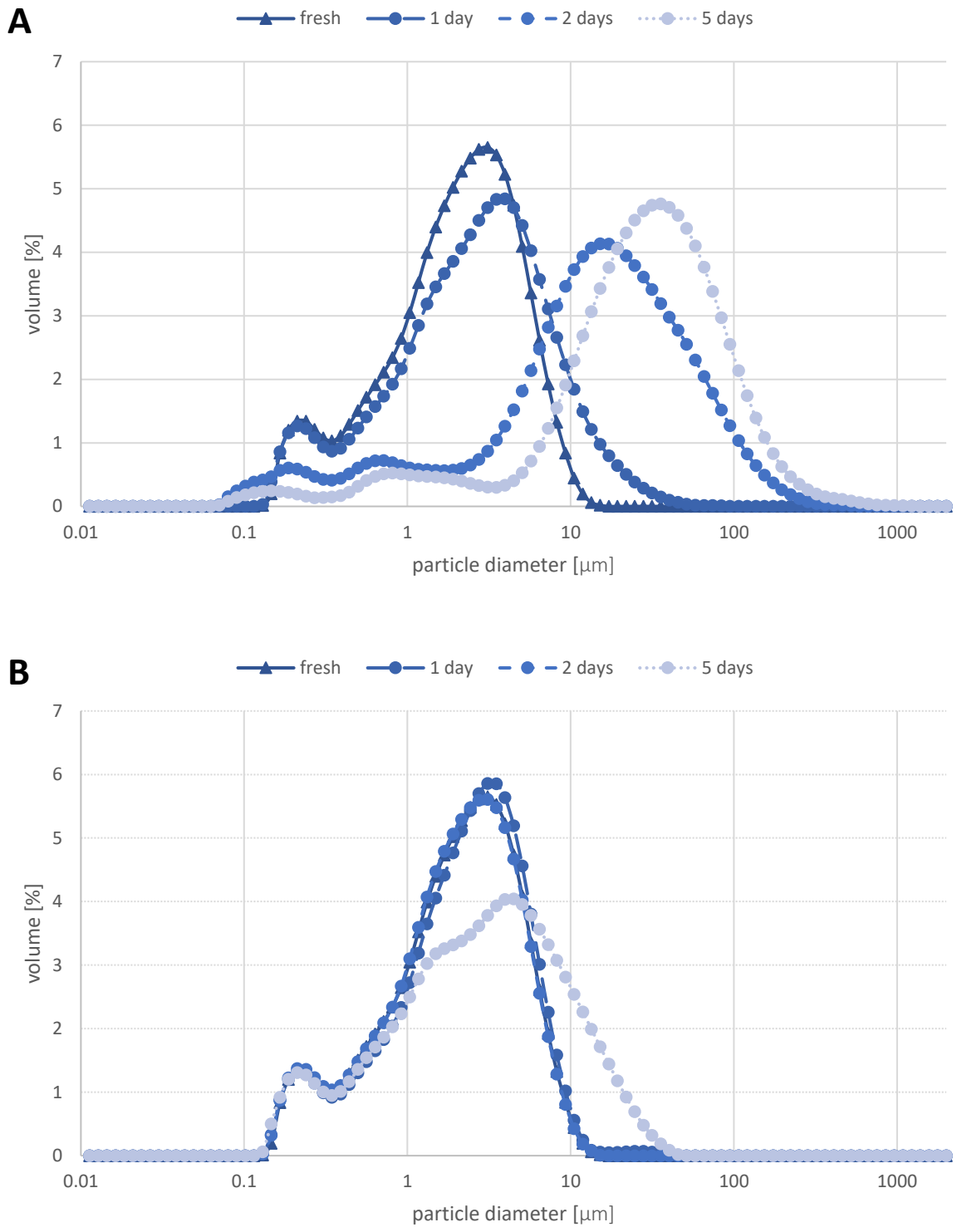

Figure 4-16. Particle size development of OBC-1. A – during storage at RT; B - during storage in a refrigerator (4 °C). Points indicated with a circle (●) have been determined as an average from measurements in triplicate, while points indicated with a triangle derive from measurements executed in quadruplicate.

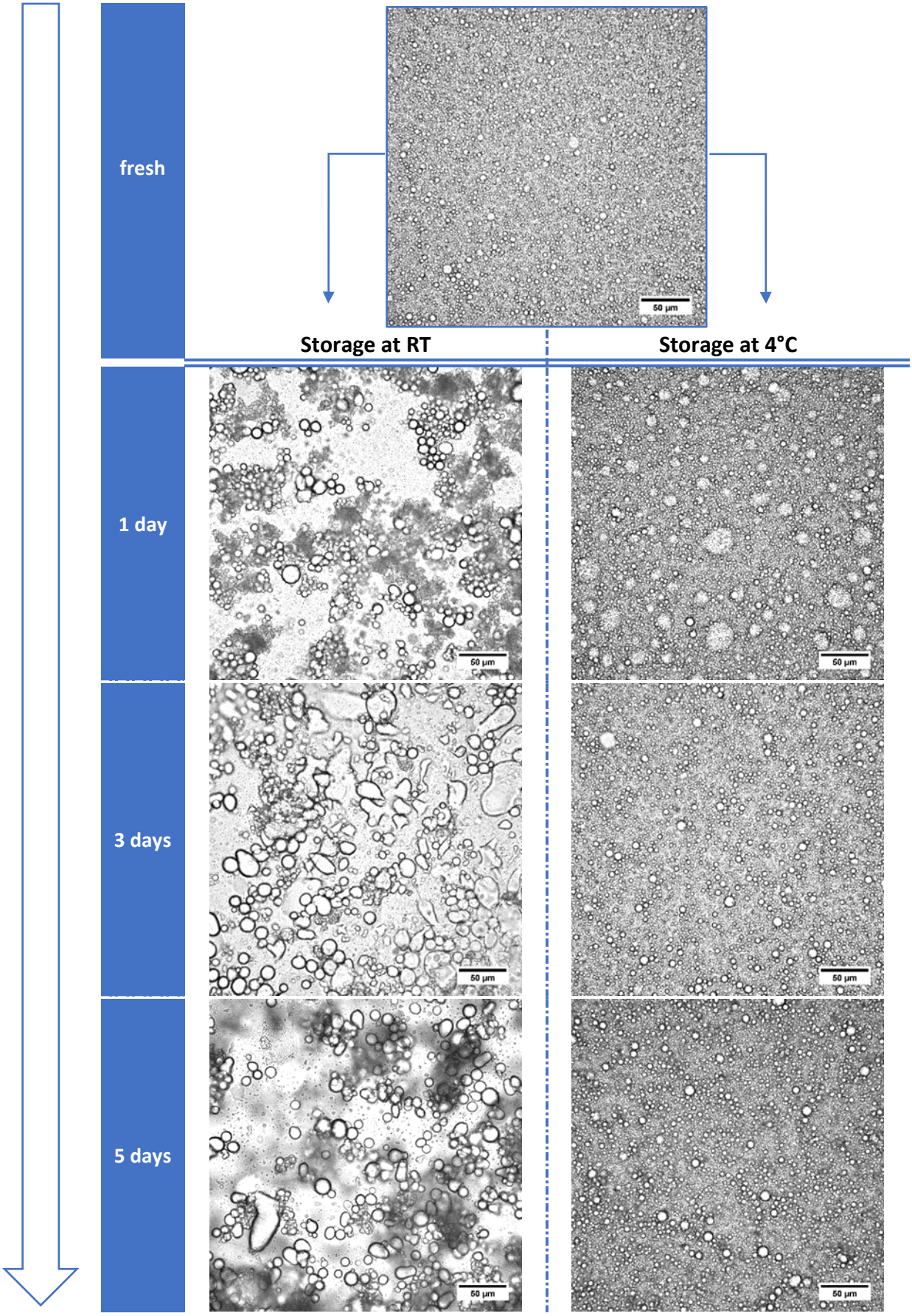

Figure 4-17. Microscope pictures of OBC-1 when stored at RT or 4°C.

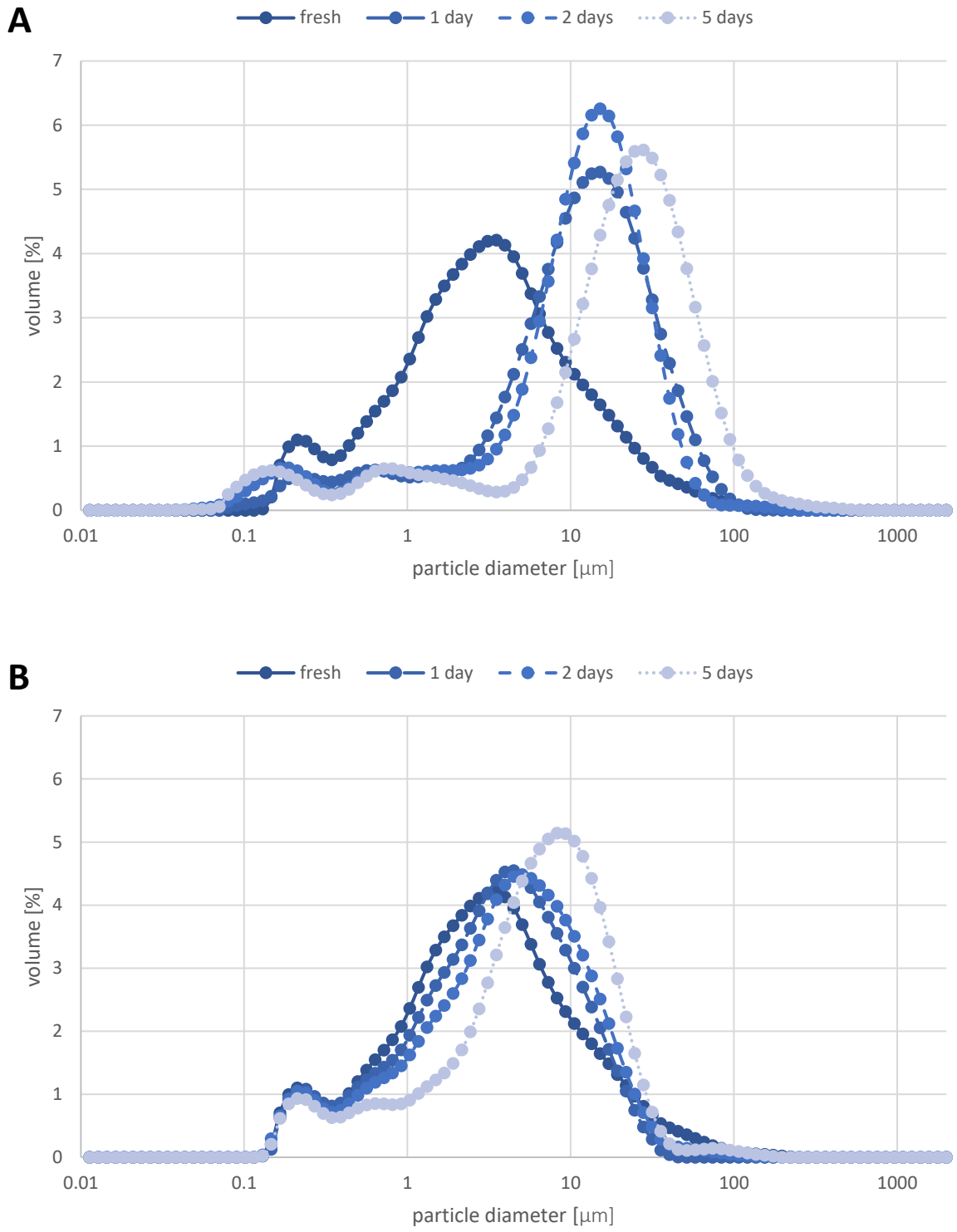

Figure 4-18. Particle size development of OBC-2. A – during storage at RT; B - during storage in a refrigerator (4 °C). All points have been determined as an average from measurements in triplicate.

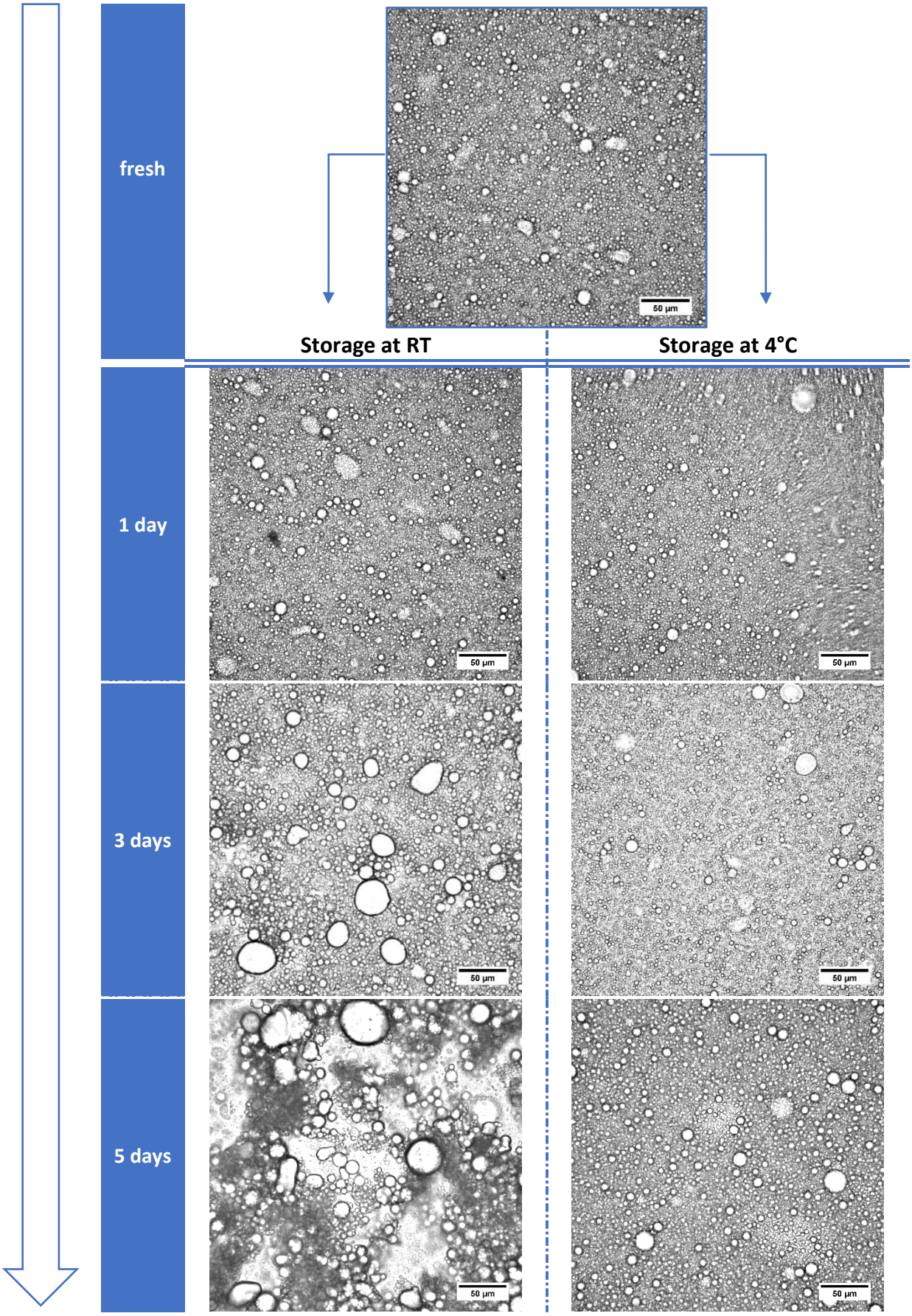

Figure 4-19. Microscope pictures of OBC-2 when stored at RT or 4°C.

4.5.1.4 Differences in size distribution among the tested fractions

In conclusion, OBD-1 is most stable regarding particle size distribution over time. The washed cream OBC-2 appears to be more stable than its predecessor OBC-1. This contradicts earlier findings reported in literature, which related an increase in stability to the presence of extraneous protein[48]. However, mentioned publication focused on oil bodies extracted from maize germ, while enzymatic activity had been suggested on soybeans. The proteins attached to an oil body surface differ depending on the raw material to be extracted and proteins attached to tomato seed oil bodies have not been characterized yet. Enzymatic activity is likely the reason for the increment in size of OBC-1 at RT. Significant fractions of the enzymes responsible for degradation are expected to have been removed during the washing step at pH 9, resulting in a higher conformity of OBC-2. Additionally, storage at 4 °C is likely to have slowed down enzymatic activity, which explains why all fractions were more stable at 4 °C than when they had been stored at RT. Storage pH is hypothesized to also influence the particle size development, but pH needed to be monitored more closely in order to confirm this assumption.

4.5.2 Charge

Depending on both their specific isoelectric point (IEP) and the pH of the surrounding solution, proteins can be charged positively (below IEP), negatively (above IEP), or neutral (at IEP); the charge of oil bodies will depend on the proteins attached to their surface in combination with the pH of the aqueous phase they are encountered in. Measurement of the ζ-potential gives the effective surface charge of oil bodies suspended in a solution and takes into account that charged particles from this solution might attach to the oil body surface, thereby influencing its net charge[74].

Two types of ζ-potential have been measured: ζ-potential development over a time frame of 7 days and the ζ-potential depending on the surrounding solution's pH.

4.5.2.1 ζ-potential at different titration pHs

A difference in isoelectric point was expected as the oil body dispersion still contains much protein. While the IEP of oil bodies typically lies somewhere between pH 5 and 6, those of proteins are often a bit lower (around pH 4). The IEP of the total dispersion would then be expected to be lower than that of purified oil bodies, assuming that interactions of proteins and oil bodies would not influence the charge distribution within the solution. In OBC-1, only proteins were expected that had been attached to the oil body membrane during centrifugation; for example, due to electrostatic forces. Finally, assuming a successful washing step, OBC-2 would only contain proteins implemented in the membrane of the oil bodies, but no extraneous proteins anymore.

Figure 4-20 shows the titration measurements of 100x diluted OBD-1. Titration was either executed from low to high pH values, or the other way around. This resulted in two distinct titration curves, which also led to different isoelectric points (2.56 when titration was executed from low to high pH and 4.18 when titrating from high to low pH).

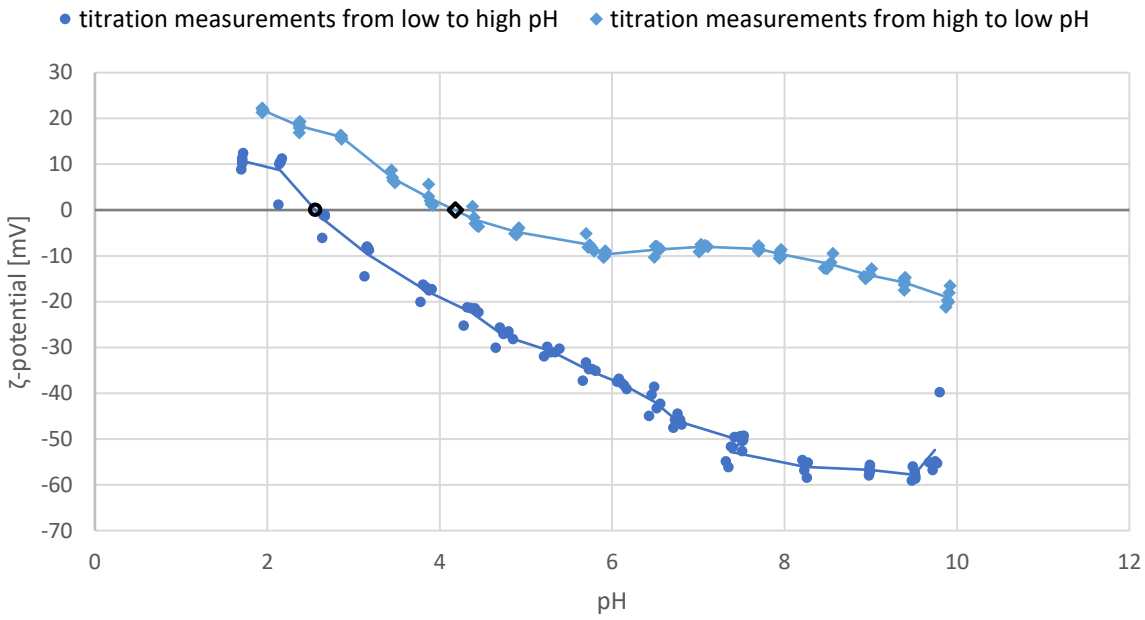

Figure 4-20. ζ-potential of oil body dispersion 1, either titrated pH 1.5-10 (rounds) or 10-1.5 (diamonds). The unfilled black shapes indicate the calculated isoelectric points (IEP).

The direction in which a titration is executed is not supposed to make any difference; a solution should have a fixed ζ-potential at a certain pH, no matter what the previous pH was. The fact that in this case two distinct curves were obtained suggests that the proteins in solution is slower in adjusting to the new pH than the measuring pace of the equipment used. With the given settings, pH was decreased or increased with about 0.5 pH units every 10 minutes (this also depended on the amount of readings that were necessary). Adjustment to a certain pH and measurement after two hours is expected to give more reliable results.

Figure 4-21 shows the autotitration curves of OBC-1. While the difference in pH values was only 1.5 in the case of OBD-1, the isoelectric points determined for OBC-1 vary by nearly five pH units (2.30 to 7.14). Commonly, isoelectric points of seed oil bodies have been reported to be in the range of pH 4-6[51,62,65]. As stated before for OBD-1, ζ-potential measurements needed to be done after storage at a specified pH for longer time to determine a more exact isoelectric point of OBC-1.

Both IEP values measured for OBD-1 were relatively low, while the titration from low to high pH resulted in an approximately neutral isoelectric point of OBC-1. Assuming the actual isoelectric point lies roughly centered between the two measured values, the isoelectric point of OBC-1 should be higher than that of OBD-1. This, in turn, suggests that OBD-1 still contains particles that lower the total charge, which are not present anymore in OBC-1. Particles that were not present in the cream layer (OBC-1) afterward must be in the subnatant phase. To assess whether those particles might indeed have influenced the overall charge of OBD-1, autotitration was executed on S-1, the subnatant of OBC-1. The resulting ζ-potential curve is shown in Figure 4-22.

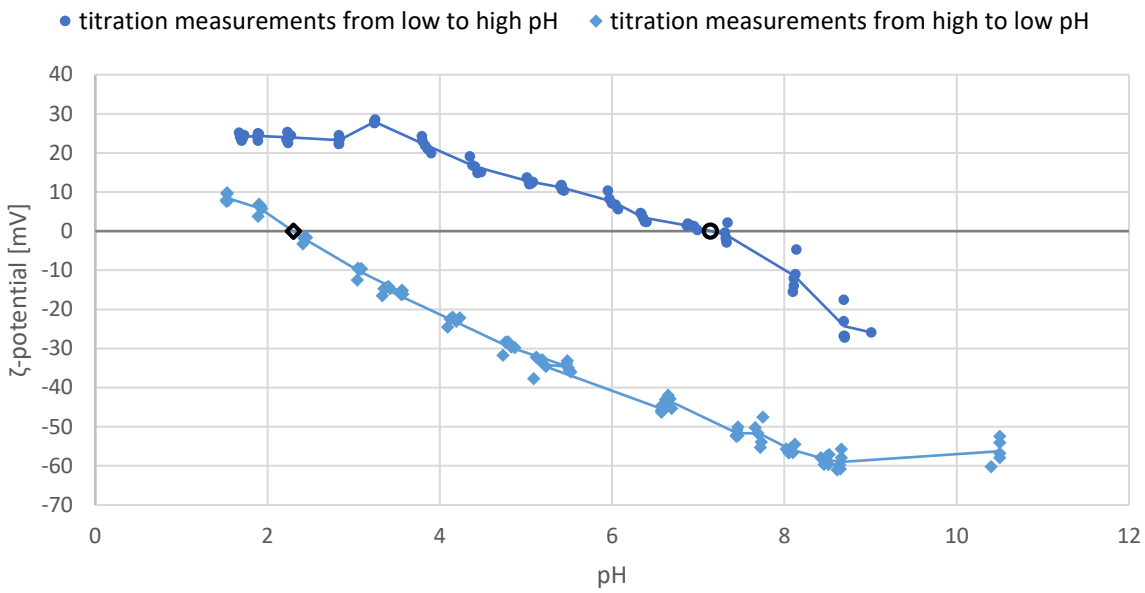

Figure 4-21. ζ-potential of oil body cream 1 either titrated pH 1.5-9 (rounds) or 10-1.5 (diamonds). The unfilled black shapes indicate the calculated isoelectric points (IEP).

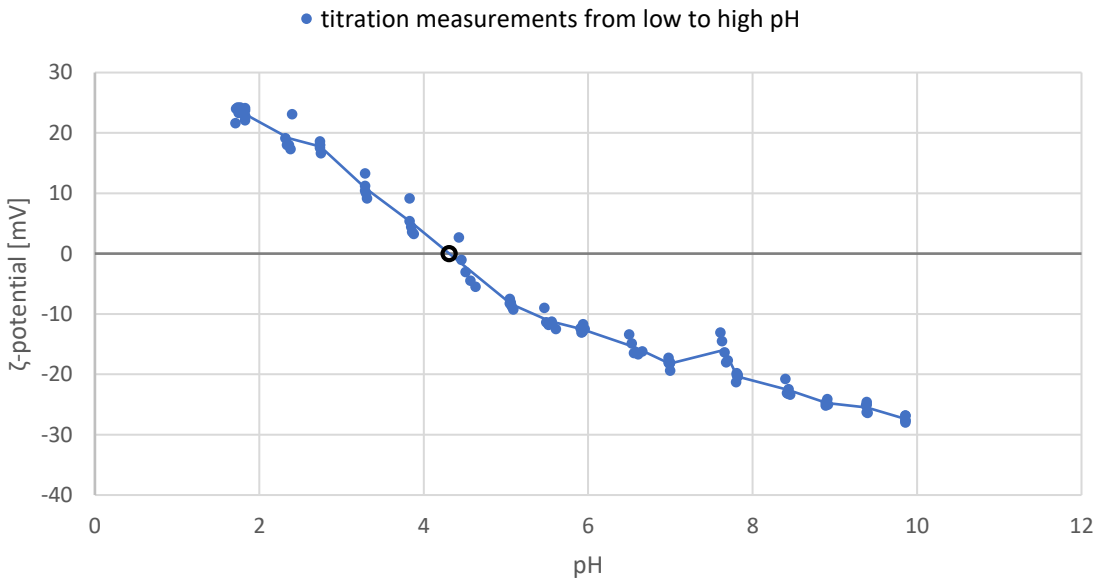

Figure 4-22. ζ-potential of the subnatants of oil body cream 1, titrated pH 1.5-10. The unfilled black shape indicates the calculated isoelectric points (IEP).

The isoelectric point of this fraction lies around pH 4, which is a reasonable IEP value for seed proteins. However, both the IEP of OBC-1 and of S-1 then lie above that of OBD-1, which is expected to be nothing else but a mixture of the former two.

The fact that the proteins contained in S-1 do not interact with the same particles anymore as they did in OBD-1 might change their overall charge. For example, it would be conceivable that hydrophobic forces led to specific formations of proteins and oil bodies in the earlier dispersion, which are changed in the subnatant fraction. A second possibility would be that the proteins measured in S-1 do belong

to oil bodies just as in OBC-1, but they are diluted differently. Dilution had been optimized for OBD-1 and was quintupled for analysis of OBC-1 and OBC-2. However, it is possible that an even higher dilution factor would have been necessary to obtain good results.

Faulty dilution would also have influenced the autotitration results of OBC-2, which can be seen in Figure 4-23. The shape represented in this figure is a typical ζ-curve. The resulting isoelectric point falls neatly into the range of seed oil bodies mentioned earlier.

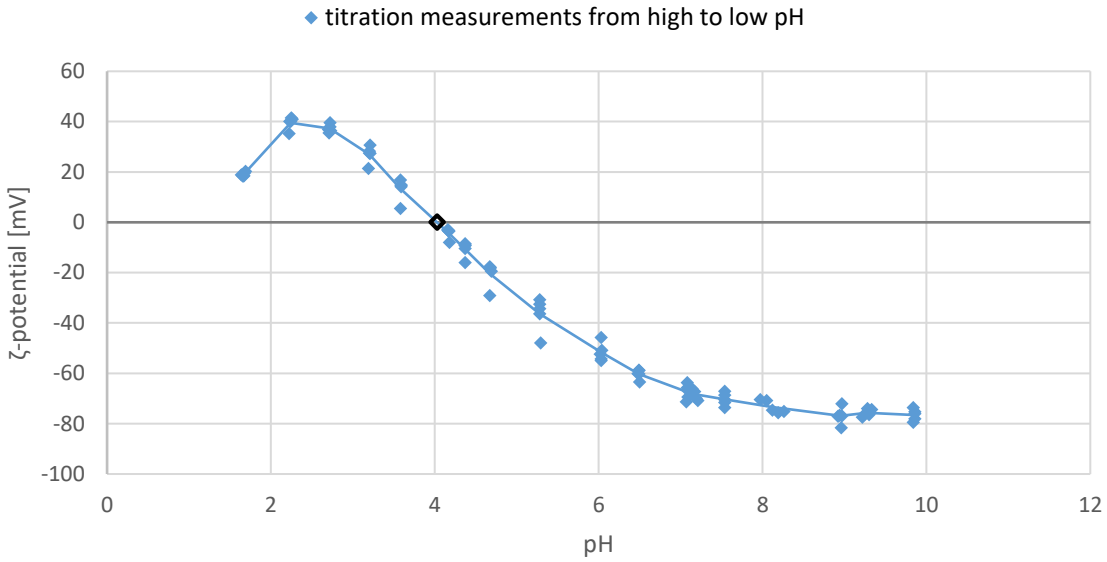

Figure 4-23. ζ-potential of oil body cream 2 titrated pH 10-1.5. The unfilled black shape indicates the calculated isoelectric points (IEP).

4.5.2.2 ζ-potential development over time

Figure 4-24 shows the development of the ζ-potential over a time frame of seven days.

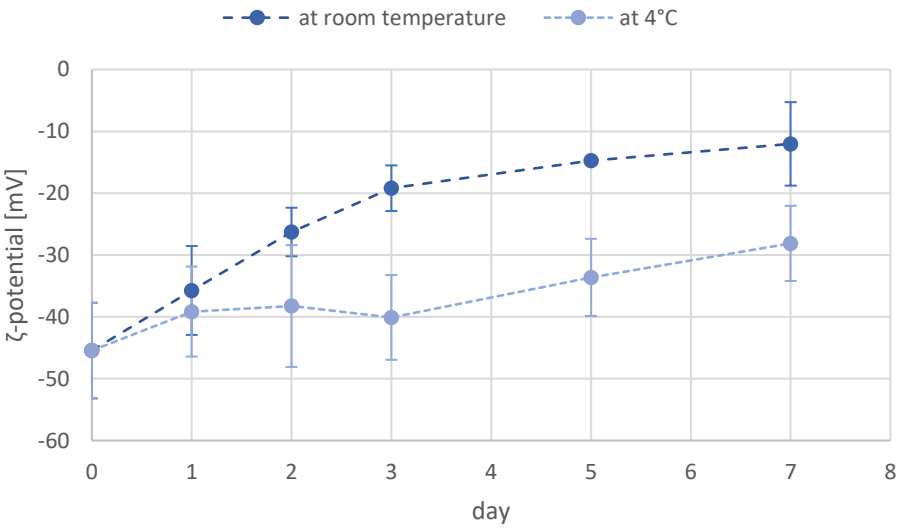

Figure 4-24. ζ-potential development of OBD-1 , 100x diluted in MilliQ water adjusted to pH 9 and stored this way at either 4 °C or RT.

Both at RT and at 4°C, the ζ-potential increases from below -40 mV towards a more neutral charge. When stored at RT, this development takes place primarily during the first three days of storage and only changes little afterward. Contrary to this, the ζ-potential of the dilutions stored at 4°C remains approximately equal during this first timeframe but starts to increase thereafter. Additionally, the difference in ζ-potential between the two storage conditions only becomes significant after three days approximately.

Hydrophobicity of the enclosed lipids might lead to clustering of the oil bodies, which could result in a change of the net charge on the surface. Similarly, proteins that might still be attached to the oil body surface might be washed into solution over the length of the storage period, changing the charge distribution on both the oil body surface and the external of the respective proteins. Dilutions had been prepared in MilliQ water that had been adjusted to pH 9 to resemble extraction conditions. On the other hand, OBC-1 was washed with demineralized water at this same pH, which resulted in an alternation of the isoelectric point by 1.5 units approximately. A more neutral charge could then be reached at a pH closer to the initially adjusted one.

4.6 Remarks on differences between OBC-1 and OBC-2

Particle size distribution, as well as visual analysis, showed that all analyzed fractions were more stable during storage at 4 °C than they were at RT. Formation of large particles suggests enzymatic degradation of the oil body membranes[62,75]. Enzymes are typically less active at low temperature, which explains why the degradation is slowed down at 4°C.

4.6.1 Particle sizes in OBC-1 and OBC-2

Enzymatic degradation only does, however, not explain why OBC-2 remains more stable than OBC-1. Additionally, the washing step of OBC-1 was expected to result in an OBC-2 being more abundant in oil, which was refuted by analysis (see Figure 4-10 on page 28). A change in pH, induced by washing, may have influenced the stability of OBC-2. Oil bodies from soybeans and peanuts were unstable close to their isoelectric point[62,66,76]. It may therefore be suggested that the different stabilities of OBC-1 and OBC-2 are based on a difference in pH, where the storage pH of OBC-1 lies close to the isoelectric point of tomato seed oil bodies. As pH was not monitored during this study, described relation remains a presumption.

The decreased oil content of OBC-2 compared to OBC-1 led to a re-assessment of the particle size measurements. From the volumetric percentage of certain particle sizes represented in 4.5.1, a *volume weighted mean* (D[4,3]) and a *surface weighted mean* (D[3,2]) were calculated, with the earlier said to be more reliable[77].

Therefore, Volume weighted means were compared between fresh OBD-1, fresh OBC-1, and fresh OBC-2. As centrifugation to obtain cream occasionally happened after fresh OBD-1 had been stored at 4 °C overnight, the volume weighted mean of these samples was assessed, too. A comparison of these values may be derived from Figure 4-25.

The volume weighted mean of OBC-2 is highest. This translates to OBC-2 being richer in large particles than the other fractions measured, which lie approximately in one range (especially when standard deviations are considered). It can additionally be observed that the particle size of OBD-1 apparently equilibrated around a mean value during storage at 4 °C overnight. The small change between OBD-1 and OBC-1 suggests that most cream particles from the dispersion arrive in the first cream layer to be formed. The fact that there is even a small decrease in volume weighted mean might refer to rather large complexes (e.g. fibers) in the dispersion. These would be separated during centrifugation and end up in the pellet rather than in the cream layer.

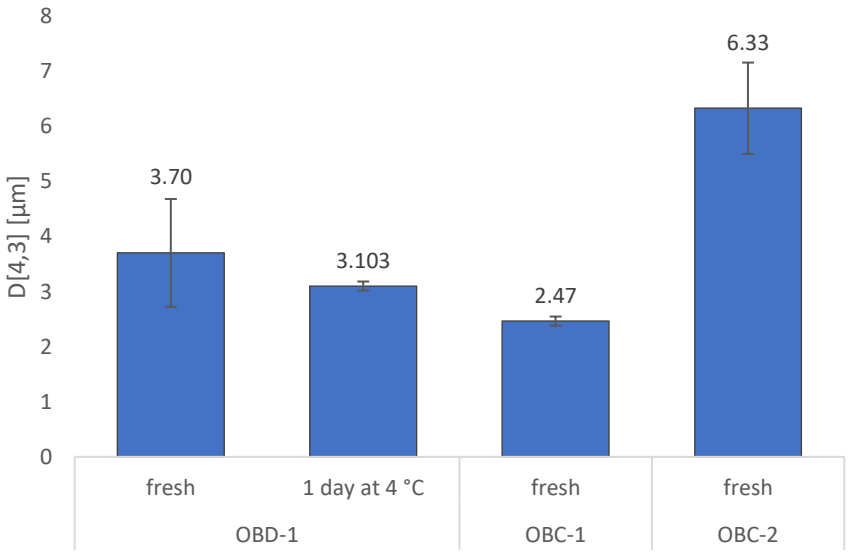

Figure 4-25. Volume weighted means of fresh OBD-1, OBC-1, and OBC-2, as well as one-day old OBD-1 as determined by the Mastersizer.

From OBC-1 to OBC-2, however, the average particle size more than doubles. This might be explained with coalescence of oil bodies due to the forces exhibited on them during centrifugation but should have been observed for OBC-1 then already, especially as both Mastersizer and microscopic analysis had shown OBC-1 to be less stable than OBC-2.

Consequently, it is hypothesized that only oil bodies of a specific minimum size are centrifuged into the top oil body cream during the centrifugation step yielding in OBC-2. Smaller oil bodies are believed to remain in the subnatants phase, from which they were discarded (see Figure 4-26).

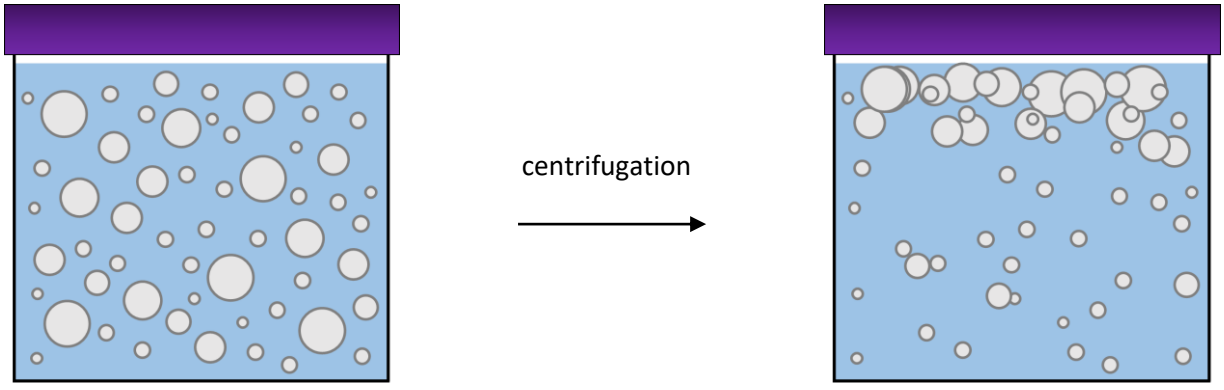

Figure 4-26. Schematic representation of the hypothesized fractionation of oil bodies on size, during centrifugation (10,000 g, 4 °C, 30 min). Only the upper layer concentrated in larger oil bodies was scooped off as OBC-2 for analysis.

In a fluid medium, two forces act on a small spherical particle: Traditionally, the dragging force F_d creates an upwards movement of the particle through friction. Contrary, the gravitational force F_g moves the particle downwards due to the difference in density. As oil bodies are less dense than the surrounding (water) phase, the effect of these forces is turned around: the gravitational force becomes negative and results in an upward movement that is hampered by the dragging force keeping it downwards.

$$\boldsymbol{F_d = 6 * \pi * R * \eta * v} \quad \textbf{4.1}$$

$$F_g = \frac{4}{3} * \pi * R^3 * (\rho_s - \rho_l) * g \quad \textbf{4.2}$$

where:

R = radius of the spherical particle (m)
η = viscosity of the liquid phase (Pa s)
v = velocity of the spherical particle (m s^{-1})
ρ_s, ρ_l = density of the spherical particle and the liquid, respectively (kg m^{-3})
g = acceleration due to gravity (m s^{-2})

Equating 4.1 and 4.2 gives the particle velocity at which the particle's vertical position does not change.

$$F_d = F_g \quad \textbf{4.3}$$

$$6 * \cancel{\pi} * \cancel{R} * \eta * v = \frac{4}{3} * \cancel{\pi} * R^{\cancel{3}2} * (\rho_s - \rho_l) * g \quad \textbf{4.4}$$

$$v = \frac{\frac{2}{9} * R^2 * (\rho_s - \rho_l) * g}{\eta} \quad \textbf{4.5}$$

4.5 is also known as *Stroke's law*[78]: the sinking velocity of a spherical particle is therefore determined by its radius, the difference in density with the surrounding liquid, the viscosity of said liquid and the g-force applied. The sinking velocity will be greater if radius, density divergence or g-force increase, but will decrease with increasing viscosity of the liquid phase.

For the case of oil bodies, the velocity will be turned upwards. The system determines G-force, densities and viscosity and consequently, the radius is the only variable. The minimum radius (R_{min}) of a spherical particle to still terminate in the cream layer can be calculated if the geometrics of the centrifuge are known[79]. In order to centrifuge also those particles that have a radius smaller than R_{min}, density difference or g-force needed to be increased. The former is difficult to achieve in the presented system, as it relies on water as an extractant and alternative liquids would still have similar properties, density being one of them. However, acceleration could be increased if suitable equipment is available.

Nikiforidis and Scholten (2015) showed that the sunflower oil volume fraction of a cream comparable to OBC-2 was highly depended on the speed applied during the centrifugation step[80]. The cream reached its maximum effective oil volume fraction of 0.91 after centrifugation at 30,000 g and 4 °C for 30 minutes, while this value was only about 0.45 (estimated from graph) after centrifugation at 10,000 g. The latter value resembles the oil content of OBC-2 presented in Figure 4-4, obtained under similar conditions.

Additionally, the authors also report an increased droplet size from the initial oil body emulsion to the final cream; while the former resulted in a surface weighted mean (D[3,2]) of 1 µm approximately, the latter expressed a value of 6 µm for this parameter. For the oil body fractions analyzed during this study, the surface weighted mean was 0.92±0.06 µm in fresh OBD-1 and 1.37±0.38 µm in fresh OBC-2. Due to these similarities and the observed consistency of OBC-2, it is likely that the produced cream resembles the high internal phase emulsion gel that has been produced by Nikiforidis and Scholten.

5 Conclusion

During the presented study, a process was developed to extract oil bodies and proteins from tomato seeds. The determined parameters for seed extraction showed little extraction efficiency when applied to dried tomato pomace. Subsequently, an enzyme-assisted aqueous extraction was executed on wet pomace, which resulted in a bright red pellet product that consisted of more than 90 wt.% water. Hygroscopic characteristics of tomato peels have been suggested to be responsible for the low volumetric percentage of oil and protein to be extracted.

Aqueous extraction from tomato seeds yielded 30.22±0.48 wt.% of the original oil in the first cream to be obtained, and still 17.31±1.19 wt.% of it were present in its washed successor, respectively. While both its oil and protein content are higher, the first cream to be obtained is unstable especially at room temperature, which was related to a possibly imperfect pH during storage or enzymatic activity degrading the oil body membranes. The second, washed cream is much steadier in composition. It remains to be elucidated which factors are responsible for this increased stability. Roughly 28 wt.% of the composition of the second cream were not characterized in detail.

It has been suggested that only oil body particles of a specific minimum diameter settle in the top cream layer, while those with a smaller diameter are lost in the underlying subnatant. Centrifugation at higher g-force might improve the yield at the cost of higher energy requirements. The effects on oil body cream stability are not clear yet. However, literature suggests the formation of a high internal phase emulsion gel with an increased oil content.

Approximately 62 wt.% of the proteins contained in tomato seeds were extracted to the aqueous phase. Only a small percentage of these proteins conclude as part of the oil body cream fractions; the majority is expected to have been discarded as the subnatant of the first cream. This subnatant has not been analyzed quantitatively during this study.

Alkaline aqueous extraction is concluded to be a valid first step in a multi-product biorefinery based on tomato seeds. Extraction and purification parameters still need to be optimized for higher yields and volumetric percentages of oil in the resulting creams. Proteins should be recovered from the respective subnatants, which could most easily be achieved by acidic precipitation. Based on ζ-potential measurements, a pH of approximately 4 is suggested.

The proposed process relies on efficient and effective separation of tomato seed and peel fractions and subsequent drying of the purified seeds. Implementation of the proposed biorefinery could be stimulated by the promotion of separated streams in the processing facilities.

6 RECOMMENDATIONS

During the presented study, only a set of parameters was assessed on a previously determined goal. These parameters still require optimization. Retrospectively, the determination of some additional parameters might have greatly improved the expressiveness of the presented results.

The pH of the tested dispersions and dilutions has not been monitored during this research but has been suggested to influence the stability of OBC-1. To better understand the interactions that might occur between oil bodies, co-extracted and possibly attached proteins and the surrounding medium, pH monitoring might be used in the future.

The washed oil body cream OBD-2 holds a lower volumetric percentage of oil than its predecessor. It is hypothesized that the centrifugation step at 10,000 g and 4 °C for 30 minutes is insufficient to separate oil bodies with a small diameter from the dispersing phase. Centrifugation for a more extended amount of time or at a higher g-force may increase the oil content in OBC-2, as suggested in literature; however, it would also raise the energy requirements and thereby related costs of the process. An optimum between oil content, oil body stability and quality of the resulting product should be determined. Based on sufficient experimental data, models for such an optimum may be developed.

Autotitration results suggest that the tested dispersions are slow in adjusting to a given pH. To receive a more reliable titration curve, pH should be adjusted overnight. Single samples should be measured, from which a titration curve may be constructed.

The subnatants of OBC-1 and OBC-2 have been analyzed on proteins qualitatively but have not been collected for the component analysis presented earlier in this chapter. Neither have the subnatants of OBC-1 and OBC-2 been analyzed on size distribution with a Mastersizer. Such measurements would provide insight into the actual fractionation of different particle sizes during centrifugation and should be executed in the future. Additionally, quantification of the extracted protein fractions will provide deeper insights into the extraction efficiency of proteins, using the applied parameters.

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PICTURE SOURCES

IKA® M20 Universal mill (in Figure 3-1): www.ika.com, retrieved on 18/12/2017.

Watering can, pipet, ice crystal, round arrow and microscope (in Figure 3-1 and Figure 4-13) from MS Office Symbols.

Trash can (in Figure 3-2): by Fahmihorizon, the Noun Project. https://thenounproject.com/search/?q=rubbish&i=1221168, retrieved 23/01/2018.

Appendix A EQUIPMENT CONFIGURATIONS

1. FREEZE DRYER

Samples were initially frozen at -18 °C for at least 4 hours. Freeze-drying took place at -80 °C in an Alpha 2-4 LD plus (Christ, Germany). Main drying lasted 48 hours after which final drying lasted an additional 24 hours.

2. MASTERSIZER

Samples were applied to a Hydro 2000SM (A) stirring at 1200 rpm, unit until an obscuration between 10% and 15% was reached. Five measurements were taken per aliquot, with a delay of 10 seconds between each of them. A refractive index of 1.46 and an absorption of 0.01 were assumed for the oil bodies, while a refractive index of 1.33 was assigned to the dispersant (water).

3. NANOSIZER

ζ-potential was measured in a disposable folded capillary cell type DTS1060/DTS1061. A refractive index of 1.455 and no absorption was applied to the oil bodies. The dispersing water phase was set at a temperature of 20 °C, viscosity of 1.0031 cP, a refractive index of 1.33 and a dielectric constant of 80.4. F(κa) was set on 1.5 based on Smoluchowski. Equilibration lasted 300 seconds, after which five measurements with 10-100 runs were taken at 20 °C. Data was analyzed according to the automatic settings of the equipment.

Appendix B PROTOCOLS AND PROCEDURES

1. SDS-PAGE

Buffer 1 (pH 8)

- 50 mM Tris-HCl
- 5M urea
- 1% SDS
- 4% 2-mercaptoethanol

Buffer 2

- 125 mM Tris-HCl
- 5M urea
- 1% SDS
- 20% glycerol
- 4% 2-mercaptoethanol

Well-visible bands can be obtained with protein concentrations around 3 mg/mL. In a 1.5 mL Eppendorf tube, weight sufficient sample to have approximately 3 mg of protein present (protein content determined with Dumas beforehand). Subsequently, add 0.5 mL of buffer one. Vortex in a … at 600 rpm for 15 minutes, then allow to rest for 10 minutes. Subsequently, add 0.5 mL of buffer 2, and repeat the vortexing and resting steps.

Heat up to 105 °C in a Eppendorf ThermoMixer C (Eppendorf, Germany), cooled down under running tab water and store at -18 °C overnight.

Before loading on the gel, execute at least one freeze-thaw cycle (thaw at RT, freeze again at -20 °C, thaw again) and spin down any remaining solids during centrifugation at 10,000 g for 1 minutes (Eppendorf 5430 R centrifuge; Eppendorf, Germany).

2. DUMAS

A calibration curve was made from the measurements taken on samples containing 1 mg, 5 mg, 10 mg, 15 mg and 20 mg of methionine. 10 mg of cellulose were used as a blank, which was run before and after the calibration curve measurements as well as every ten measurements.

10-20 µg of a dry sample were carefully weighted into an aluminum container that was crumbled into a little ball afterward. Dumas analysis was done in triplicate with the aid of a FlashEA 1112 NC Analyzer (Thermo Fisher, United States). A conversion factor of 5.7 was used to convert from nitrogen to protein content[65].

Appendix C SUPPLEMENTARY DATA

Table A 1. Amino acid profile of tomato seeds as determined by various authors. P = protein, PE = protein extract, LPC = lipid-protein concentrates. Essential amino acids are marked with an asterisk ('*'). Concentrations that have been calculated from amino acid concentrations to the total sample and amino acid concentrations to the total sample are marked with a plus-minus sign ('±')

Author	Tsatsaronis & Boskou[25]	Brodowski & Geisman[81]	Latlief & Knorr[56]		Tchorbanov et al.[30]	Persia et al.[54]	Sarkar & Kaul[55]
Year	1975	1980	1983		1986	2003	2014
Seeds origin	*Greece*	*United States*	*United States*		*Bulgaria*	*United States*	*India*
	g/100 g P	g/100g P±	g/100g P	g/100g PE	g/100g LPC	g/100g P±	g/100g P
Lys*	4.94	6.45	5.50	3.36	4.10	5.39	5.96
His*	2.2	2.38	2.19	1.68	2.40	2.22	2.50
Arg	8.83	9.92	10.00	11.34	7.10	8.63	
Asp	9.58	11.95	10.61	8.82	10.60	10.48	
Thr*	3.01	3.53	3.43	3.36	5.00	3.29	3.65
Ser	4.98	5.33	4.86	4.20	6.95	4.93	
Glu	18.49	18.99	20.95	24.37	17.60	18.79	
Pro	5.39	5.50	3.73	3.36	3.80	5.52	
Gly	4.64	4.86	3.70	5.04	8.95	n. a.	
Ala	3.72	4.21	3.40	4.62	8.35	4.51	
1/2 Cys	0.6				Traces		in SAA
Cys		1.65	1.05	1.26		1.62	in SAA
Val*	3.7	4.15	3.64	2.94	4.80	4.38	5.52
Met*	0.78	1.62	1.63	1.68	2.10	1.58	in SAA
Ile*	3.52	3.74	3.23	2.52	4.05	3.90	4.93
Leu*	5.86	6.15	5.82	5.88	6.75	6.20	7.79
Tyr	3.38	4.86	3.95	2.52	2.65	3.64	in AAA
Phe*	3.64	4.71	4.26	4.20		4.63	in AAA
Trp*	0.95		0.16			n. a.	1.24
SAA[a]	0.78	1.62	1.63	1.68	2.10	1.58	3.06
AAA[b]	10.17	11.95	10.56	8.40	5.05	10.48	8.73

[a] Sulfur containing amino acids (methionine, cysteine)
[b] Aromatic amino acids (phenylalanine, tryptophan, tyrosine, histidine)