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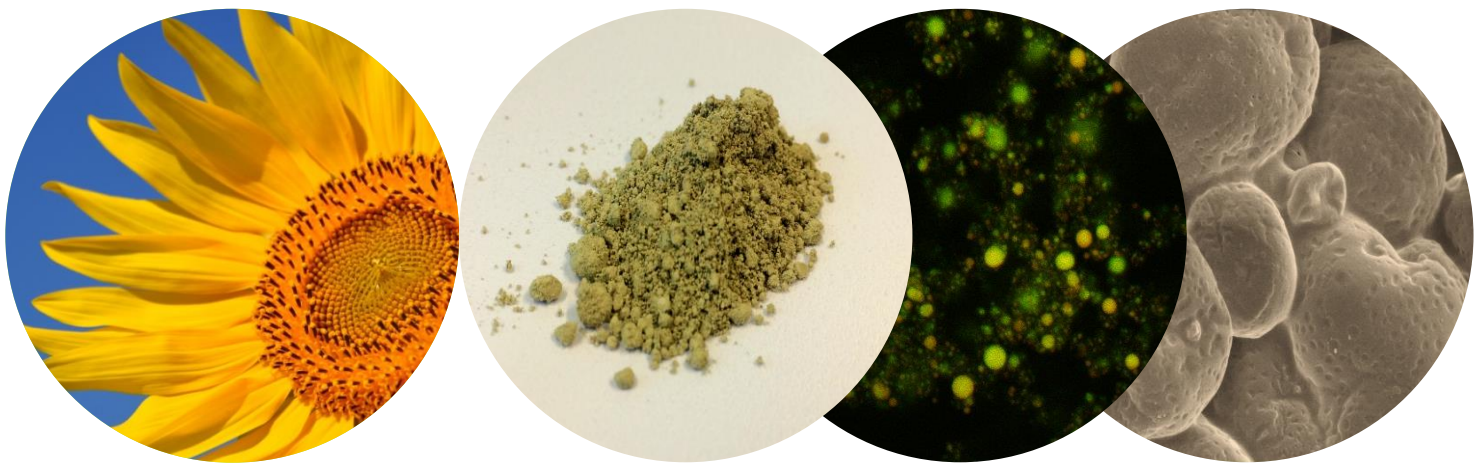
MSc Thesis Biobased Chemistry and Technology

Sunflower-based protein fractions for food applications

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Sunflower-based protein fractions for food emulsions

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Preface

Writing this thesis at the Biobased Chemistry and Technology department was a great experience. I have improved my research skills and became confident in applying the knowledge acquired during my Bachelor and Master in Food Technology.

I would like to thank Dimitri Karefyllakis for his patience in helping and answering all my questions. He always encouraged me to do more experiments in one week than I thought was possible, which was essential for the completion of my thesis in the planned time period. His knowledge and critical reflection was very helpful during the writing of my thesis. I would also like to thank Costas Nikiforidis for his open door policy were I could always walk in and ask questions. I owe special thanks to Jos Sewalt and Maurice Strubel for introducing me to all lab equipment and always helping me during my experiments. At last, I would like to thank Elinor Scott for being my examiner and taking the time to read my report.

Abstract

Environmental concerns caused by a growing world population lead to a search for alternative food sources and production processes. Characteristic examples of sources like these can be found in agro-industry where large amounts of materials are wasted despite their high nutritious content. An example of the inefficient use of materials is sunflower press cake, which is a by-product of the sunflower oil currently not valorised for human consumption. The cake cannot be used for human consumption due to undesired reactions that are caused by the conventional way of processing the cake into pure protein isolates. To produce isolates excessive heating steps, organic solvents and acidic conditions are used that destroy the original functionality of the compounds.

The combination of cold pressing and aqueous fractionation is a promising sustainable substitute to the conventional process as it excludes the heating steps, organic solvents and acidic conditions. The result is not a protein isolate, but a fraction consisting of proteins, lipids and phenolic compounds. It is suggested that the interactions between those compounds have positive effects in food applications. Therefore, this research puts the emphasis on functionality instead of purity by producing fractions rather than isolates.

The effect of centrifugation force (2000, 6000, 8000, 10000 and 14000 xg) on the composition of fractions was tested. The force did not affect the protein content within the fractions. In contrast, a sudden decrease of lipid content was observed at forces higher than 8000 xg. At higher forces a better separation of phases is realised, which probably resulted in the oil cream layer being removed by filtration. Therefore less lipids were observed in the fraction. An increase of the fibre content at higher forces could not be explained.

After composition analysis, the structure was analysed using confocal light scattering microscopy and scanning electron microscopy. Emphasis was placed on the lipid particles as it is not known if they are present in original oil bodies or in stabilized oil droplets. Structure analysis showed a network between proteins and lipid particles within the fraction that was absent after the removal of fat. It was proven with CLSM and SEM that part of these lipid particles are native oil bodies after comparing the images with oil body powder and defatted fractions. The

structure analysis of the fractions led to the conclusion that native oil bodies were among the components within the fractions isolated with aqueous fractionation.

The interfacial properties of the fraction were determined using automated droplet tensiometry. The interfacial tension of the fractions (10 mN/m) was lower than that of the defatted fraction. This suggests that the oil bodies present in the fraction strengthen the stability of the oil in water emulsion, thereby implicating interactions between compounds in the fraction are beneficial. Sunflower-based protein fractions can therefore be seen are promising materials for food applications.

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1. Introduction

The global demand for agricultural production and resources is increasing due to the growing world population and prosperity. Agriculture is concurrently degrading the environment by depleting land, energy and water resources. An increase of food production and a decrease in agricultural impact on the environment is necessary to guarantee food security for the future world population [1, 2].

As a result of the growing world population the protein demand is increasing. Proteins are one of the most important components within the human diet [3]. A distinction is made between plant-based and animal-based proteins. Animal proteins require more agricultural resources such as land and water. In addition, the conversion of feed protein to animal protein is low [4]. These environmental concerns have increased the interest in using plant proteins [5-7].

In general, plant proteins are derived from soy beans [8]. Soy beans do not grow in Northern Europe and therefore depend on long supply chains, which is of environmental concern. An interesting alternative is the use of sunflower seeds. Sunflower seeds are a promising source of proteins and can be grown in a moderate climate area as Northern Europe [5, 9].

Sunflower seeds are mainly grown for sunflower oil production [10]. The by-product of the sunflower oil industry is a sunflower press cake. Sunflower cake is currently not valorised for human consumption [11, 12]. This is due to (1) protein denaturation caused by the harsh conditions during oil extraction and (2) covalent interactions of phenolic compounds with proteins present in the cake [13, 14]. Oxidation of these compounds to quinones negatively affect protein solubility and functionality [15, 16] or decrease digestibility [17, 18]. This has led to sunflower cake being used as animal feed. However, this cake can be valorised since it consists of valuable components like proteins, lipids, phenols and carbohydrates [5, 11]. Using ingredients from by-products as sunflower cake for human nutrition instead of animal feed is a promising approach to increase the sustainability of food production [19].

Plant protein isolates (PI) are currently obtained from sunflower seeds using a purification process [9, 20, 21]. Companies combine pure ingredients as PI to guarantee quality and composition of end products [7]. Processing conditions as

temperature, the addition of organic solvents and pH are harsh in order to obtain a pure protein isolate. These conditions are responsible for denatured PI and brown or green coloured protein powders [20], which are of lower functionality in food applications [5, 7, 22]. Excluding harsh conditions could result in functional proteins obtained by a more environmental friendly process.

Cold pressing followed by aqueous fractionation is a promising substitute to the conventional processing of sunflower seeds [23, 24]. No excessive heating, organic solvents or non-neutral pH values are used to prevent protein denaturation and protein-phenol reactions [15]. Instead of obtaining a pure protein isolate this process results in a fraction. This fraction mainly consists of proteins, but also other components as lipids, phenols and carbohydrates are present. Research on other oil seeds has shown that fractions potentially have similar or better functionalities than PI [7, 22, 25-28]. The aim of this research was to gain more insights into the functional properties of sunflower-based protein fractions by studying its process, composition, structure and applications.

1.1. Production process

The conventional process of the valorisation of sunflower cake focusses on purity. From sunflower cake isolated components are extracted and subsequently combined to obtain a final product. In contrast, the fractionation process consisting of cold pressing and aqueous fractionation focusses on functional fractions rather than pure components. These functional fractions consisting of multiple components can potentially be combined into the same final product (Figure 1) [29].

The findings of Pringent et al. [15] and von Staszewski et al. [30] support this mild processing to functional fractions. It was found that phenols and especially phenol-protein complexes enhance functionality. Ishii et al. [31] and Deleu et al. [32] found that the lipids present in sunflower seeds enhance functionality as well. These findings suggest that the compounds in sunflower-based protein fractions cooperate positively. Therefore, the use of fractions obtained from sunflower press cake for food applications is promising.
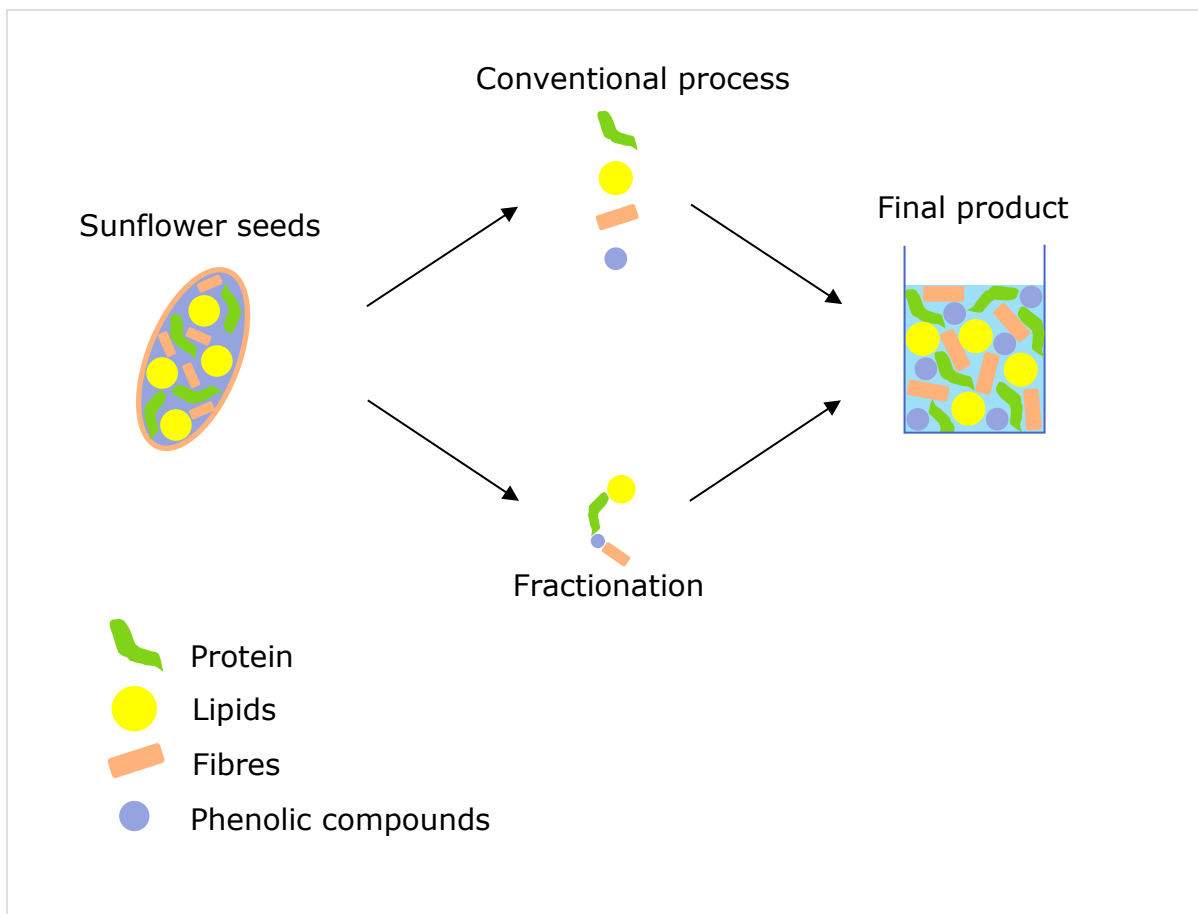
Fig. 1. Schematic representation of the conventional and fractionation process of sunflower cake.

1.1.1. Cold pressing

The conventional process is focussed on maximal oil extraction from sunflower seeds (*Helianthus annuus* L.) [33]. The composition of the residue, a cake consisting of high amounts of protein (29 - 35%) is shown in Table 1 [11, 12].

Table 1. Composition of sunflower cake and seeds [5, 11].

Component	Sunflower seeds (wt%)	Sunflower cake (wt%)
Proteins	10 – 27.1	29.4 - 35
Lipids	34 - 55	1.3 – 3.5
Carbohydrates	18 - 26	38.3 – 45.6
Phenolic compounds	1.1 – 4.5	7.2 – 7.8
Minerals	2 - 4	
Ash		7 – 7.2

The oil is obtained after defatting sunflower seeds by excessive temperatures during pressing and the use of organic solvents [5, 34, 35]. An example of a solvent used in oil extraction is hexane. Hexane is an explosive and nerve-

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damaging solvent, which has led to safety concerns during oil production and the need for more environmentally friendly extraction solvents [36, 37]. The use of organic solvents and heating steps also results in a decrease in oil quality and the loss of native structures of proteins, which decrease functionality [22, 27, 38, 39].

In contrast, no heating steps and organic solvents are used during cold pressing [22]. These mild conditions result in less oil being extracted [40, 41], but instead more functional proteins are present in the cake [7]. Figure 2 shows the differences between conventional pressing and cold pressing.
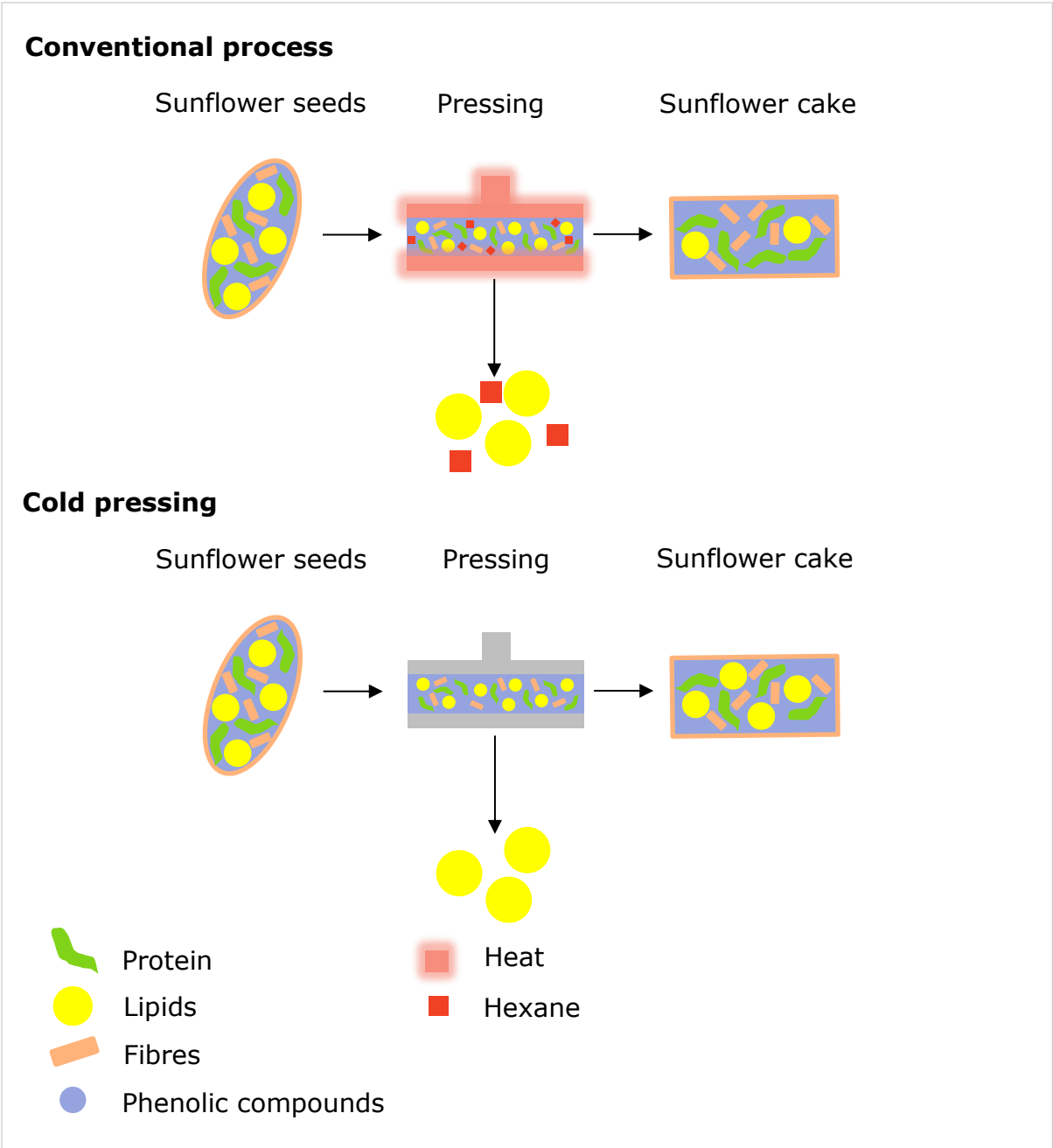

Fig. 2. Schematic overview of the conventional process compared to cold pressing.

1.1.2. Aqueous fractionation

Conventional processes are focussed on producing PI from oilseed cake. Processing includes dissolving the cake in water and changing the pH value to precipitate the proteins at their isoelectric point [42]. A pH value close to the isoelectric point of proteins causes denaturation, which negatively affects the functionality of PI [5, 22, 25, 43]. PI is obtained after spray drying, which is necessary to keep PI stable over a long time [7, 44].

Instead of the conventional isolation procedure of plant proteins, it seems that it would be more beneficial and pragmatic if sunflower protein fractions would be produced according to yellow peas aqueous fractionation found by Geerts et al. [25]. During this research, peas were milled and dispersed in water at pH 6.8 (ratio 1: 8.8). No different pH values were used in order to conserve advantageous native functionalities. After dispersing the cake in water the solution was centrifuged twice to obtain a protein rich soluble fraction [25].

The same process can be applied for the processing of sunflower seeds. Using a neutral pH prevents denaturation of proteins and also prevents the oxidation of phenolic compounds to quinones, which makes sunflower fractions suitable for human consumption [15]. Aqueous fractionation of sunflower seeds would therefore focus on the potential enhancement of protein functionality through protein-phenol interactions instead of producing a highly pure but altered protein isolate [7, 14, 26]. Figure 3 shows the differences between conventional pressing and aqueous fractionation.

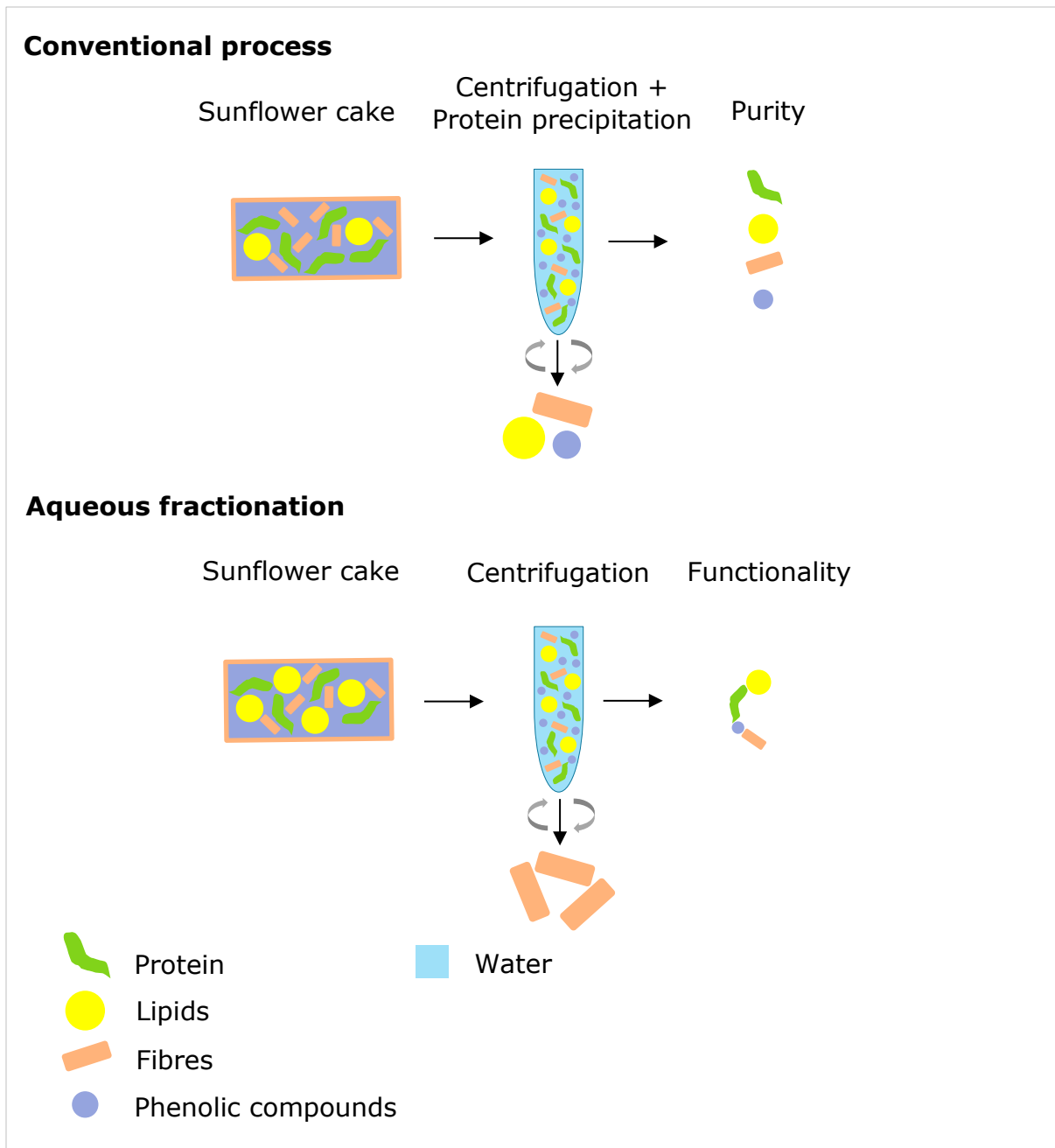**Fig. 3.** Schematic overview of the conventional process compared to aqueous fractionation.

1.2. Composition

The main and most important components of fractions obtained by aqueous fractionation are proteins, fat and phenolic compounds [26, 45].

Proteins

Proteins present in sunflower seeds can be distinguished in two main classes. Together they cover 95% of the total protein content present in sunflower seeds [5]. The first class is represented by the 11-S globulins, which molecular weight ranges between 300 and 350 kDa. 70% of the total protein content consists of 11-S globulins. The second class is 2-S albumins, which covers 30% of the total protein content. The 2-S albumins have molecular weights ranging between 10 and 18 kDa [14, 46-48]. These two classes of sunflower proteins denature at temperatures higher than 70 °C [5, 49]. Ramos Sepulveda (2017) [29] has found the presence of these proteins in sunflower-based protein fractions.

Oil bodies

Lipids in sunflower seeds are stored in natural organelles known as oil bodies [50]. Oil bodies consist of triacylglycerides surrounded by a layer of phospholipids that are embedded in proteins (Figure 4). These proteins are oleosins, caleosins and steroleosins, which are a third small group of proteins also found in sunflower seeds (around 20 kDa) [46, 51]. This protein layer prevents the oil bodies from coalescing or flocculating [52-54]. The size of sunflower oil bodies is around 1 μm and they are spherically shaped [36, 52, 55]. It is unknown if these oil bodies are still present in the fractions after the fractionation process.
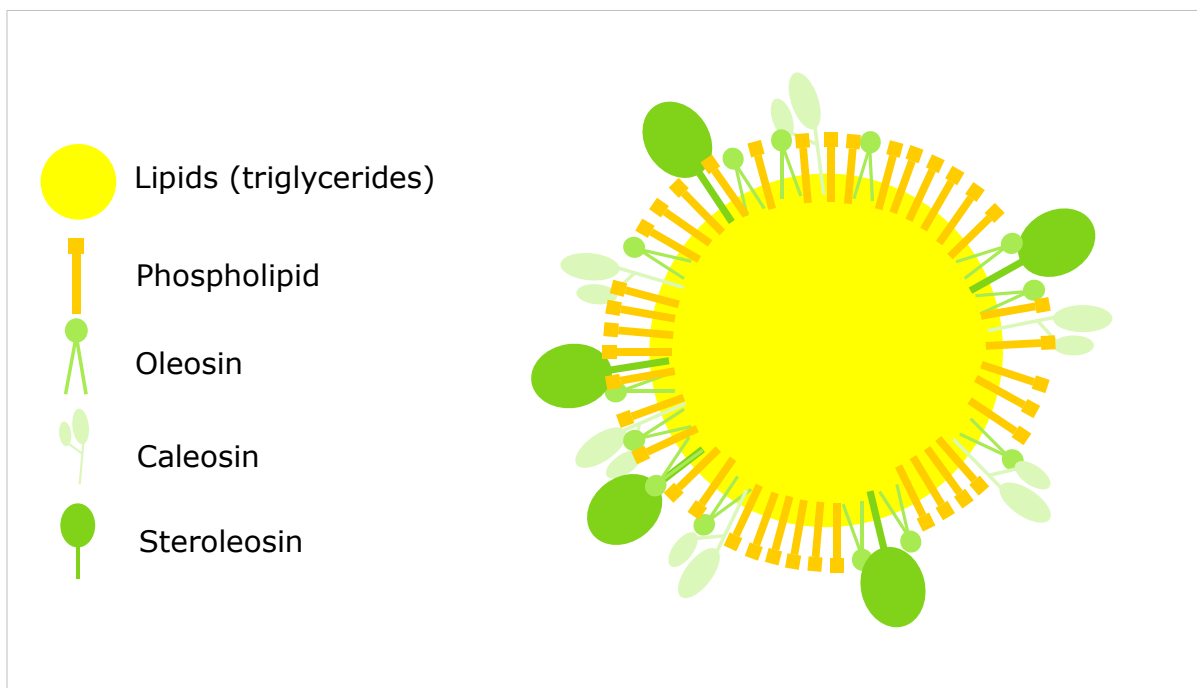

Fig. 4. Schematic representation of an oil body [56, 57].

Phenolic compounds

The phenolic compound content of sunflower seeds is maximum 4.5 wt% as is reported in Table 1. Phenols are good antioxidants and have antibacterial and anticarcinogenic properties and are therefore interesting components within the fraction [26, 58]. Chlorogenic acid (CGA) is the major phenolic compound in sunflower seeds as it represents 70% of the total phenolic content [59]. CGA is an ester of caffeic and quinic acid with a molecular mass of 354 kDa. CGA can be oxidized to quinones at alkaline and acidic pH. Oxidation of phenols leads to brown or green colouring of protein powders and negatively affect the functionality of proteins [20]. At neutral pH CGA is relatively stable and probably no oxidation will occur [15].

1.3. Structure

As explained in the last chapter previous research has not investigated the presence of oil bodies in sunflower fractions after cold pressing and aqueous fractionation. However, the structure of oil bodies is studied thoroughly in previous literature [60]. Confocal light scattering microscopy (CLSM) images show a clear spherical shaped oil body surrounded by a layer of oleosins [53, 61]. Oil bodies in relation to extraneous proteins is studied as well [52, 62]. But, this previous literature is based on oil body fractions with a fat / protein mass ratio around 5:1. Fractions obtained after aqueous fractionation are expected to consist of at least 50 wt% proteins and less fat [25].

1.4. Applications

An example of an application for sunflower-based protein fractions is an emulsion. An emulsion is a dispersion of oil droplets in water. In time, these oil droplets coalesce or flocculate and subsequently oil and water are separated into different phases. In most food products this destabilization of oil droplets is undesirable. To prevent destabilization surface-active molecules are added to the oil in water emulsion. Surface-active molecules are used as emulsifiers by forming membranes around the oil droplets thereby preventing them from coalescing or flocculating [5-8].

Proteins are one of the most commonly used surface-active molecules able to stabilize emulsions against phase separation of oil and water during storage (Figure 5.1) [47, 63-65]. Oil body isolates can act as surface-active molecules as well [31, 62, 66]. Having residual oil in the sunflower press cake is therefore a potential advantage. The surface-active capabilities of oil bodies suggest that oil bodies within a fraction can enhance the functionality of the fractions. Cold pressing would not only be beneficial to the functionality of proteins, but also to the functionality of fractions (Figure 5.2).
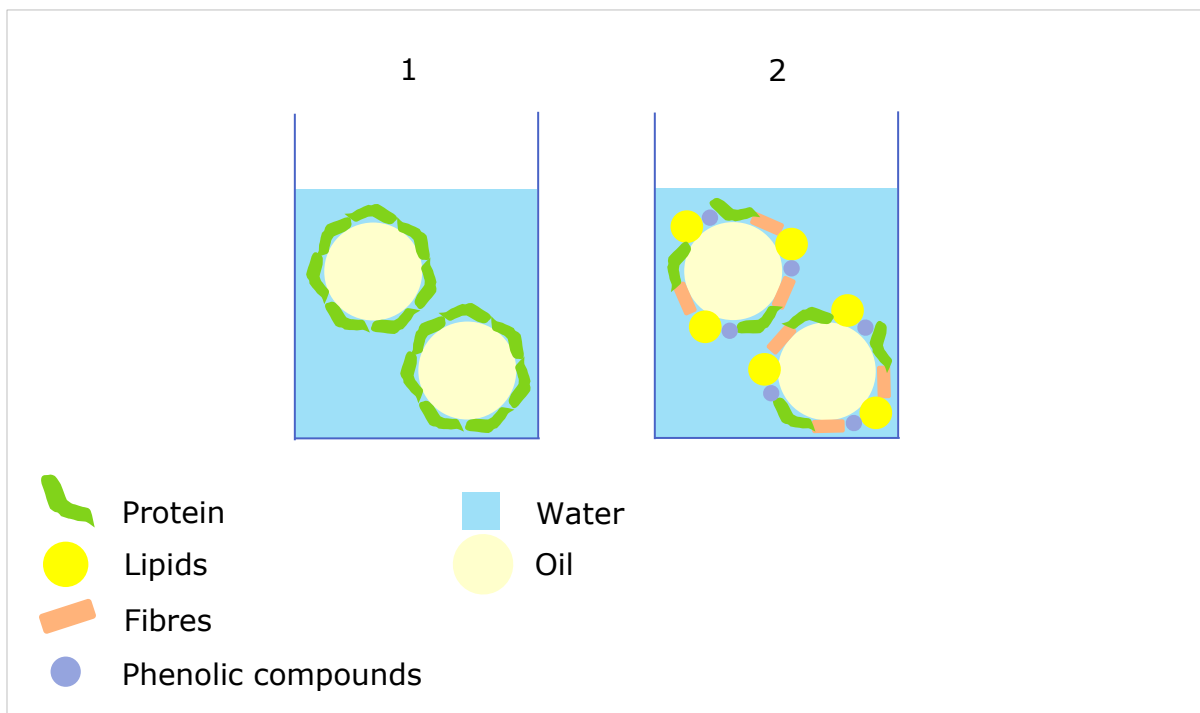

Fig. 5. A schematic overview of emulsions made with protein isolate and with fractions.

Karefyllakis et al. [26] has found that protein-phenol complexes positively affect the stability of an emulsion. Protein isolates were mixed with CGA at different ratios (1:1, 1:5 and 1:10) and tested on their interfacial tensions. Complexes had lower interfacial tensions than the protein isolate indicating a better stability of the emulsion. This suggests that functional fractions are promising substitutes to the less environmental friendly isolates. Geerts et al. [25] and Pelgrom et al. [28] studied the fractionation of yellow peas. A protein fraction (~60%) and a starch fraction (~65%) were tested on their emulsifying and gelling behaviour. It was shown that these fractions were able to prepare stable emulsions and firm gels due to the interactions between the different components. These emulsions and gels were at least of similar quality compared to emulsions and gels made from

protein and starch isolates. These findings promote the full valorisation of sunflower cake as previous literature has shown that applications made with functional fractions seem at least comparable to those made from isolated components.

Problem statement

As mentioned before the valorisation of sunflower cake to fractions is promising. Current research is mainly focussed on the fractionation of other sources [7, 9, 19, 22, 25, 27, 28, 38]. Therefore, this research investigates the fractionation of sunflower seeds. It starts with how the conditions of cold pressing and aqueous fractionation affect the composition of the cake and fractions (Chapter 3.1.). Different centrifugal forces are tested in relation to composition. This composition will be taken into account when analysing the structure of the fractions (Chapter 3.2.). Knowledge of the structure characteristics of fractions is needed to understand how processing steps affect the components extracted from the seeds. For example literature is not consistent in the presence of original oil body organelles after processing [60, 62, 67]. Interfacial properties (Chapter 3.3.) of the fractions will be taken into account to get more insights on how fractions behave at the oil water interface. This behaviour is related to possible applications of sunflower-based fractions.

Research objectives

The aim and scientific relevance of this research was to gain more insight into sunflower-based protein fractions for food applications. The following research objectives were used:

1. Preparation of a sunflower press cake.
2. Preparation of fractions with different centrifugal forces.
3. Investigation of the composition of fractions.
4. Characterisation of the structure of fractions.
5. Characterisation of the interfacial properties of fractions.

2.   Materials and methods

2.1. Materials

Sunflower seeds were purchased from Cargill (Cargill, The Netherlands) and AH BASIC Zonnebloemolie was bought at an Albert Heijn store (Albert Heijn, The Netherlands). Petroleum ether (40 – 60 °C), ethylenediaminetetraacetic acid (EDTA) ($C_{10}H_{16}N_2O_8$), sodium hydroxide (NaOH, 1 M and 0.1 M), hydrochloric acid (HCl, 37% v/v), methanol (CH_3OH), acetonitrile (ACN) (C_2H_3N), trifluoroacetic acid (TFA) ($C_2HF_2O_2$), sodium dodecyl sulphate (SDS) ($CH_3(CH_2)_{11}OSO_3Na$), Tris base ($C_4H_{11}NO_3$), glycine ($C_2H_5NO_2$), bromophenol blue (BPB) ($C_{19}H_{10}Br_4O_5S$), 2-mercaptoethanol ($HSCH_2CH_2OH$), aluminium oxide (Al_2O_3), nile red ($C_{20}H_{18}N_2O_2$), ethylene glycol ($C_2H_6O_2$) and liquid nitrogen were purchased from Sigma Aldrich (Sigma, USA). Glycerol ($C_3H_8O_3$) was bought from Acros Organic (Acros, USA) and natrium dodecyl sulphate ($NaC_{12}H_{25}SO_4$) was supplied by Merck (Merck, Germany). For dialysis a membrane SnakeSkin® Dialysis Tubing, 10 K MWCO (Thermo Fischer Scientific, USA) was used. 1 μ-Slide 8 Well ibiTreat was bought from Ibidi (Ibidi, Germany). Unless stated otherwise, all experiments were carried out at room temperature and in duplicates. In all experiments MilliQ water was used, with the only exception of dialysis where demi water was used.

2.2. Methods

Figure 6 gives a schematic overview of all processing steps and conditions used in this research to develop the sunflower-based protein fractions. This method is explained in detail in chapter 2.2.1. and 2.2.2.
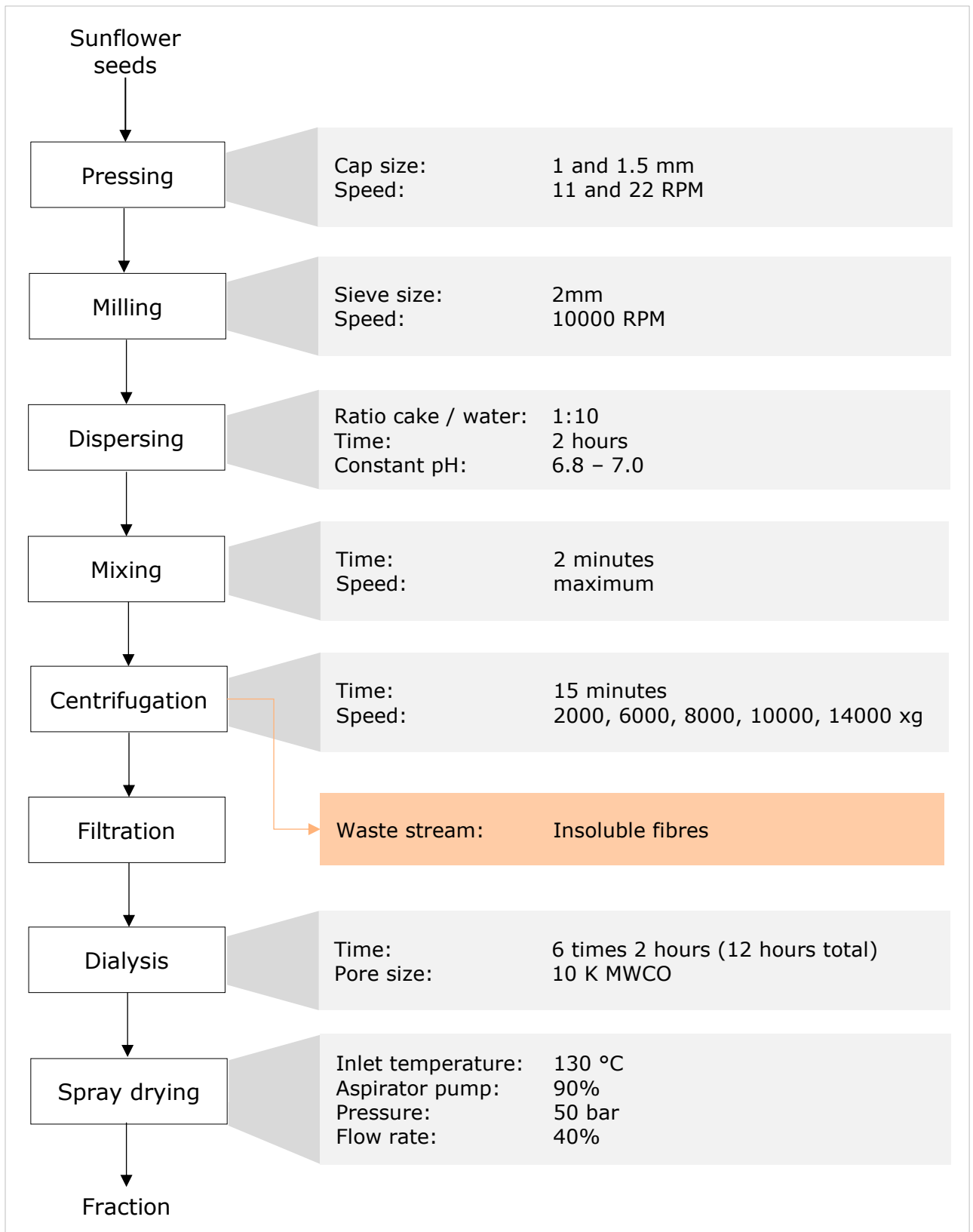
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Sunflower
seeds
  │
  ▼
┌─────────────┐      Cap size:        1 and 1.5 mm
│  Pressing   │      Speed:           11 and 22 RPM
└─────────────┘
  │
  ▼
┌─────────────┐      Sieve size:      2mm
│   Milling   │      Speed:           10000 RPM
└─────────────┘
  │
  ▼
┌─────────────┐      Ratio cake / water:   1:10
│  Dispersing │      Time:                 2 hours
└─────────────┘      Constant pH:          6.8 – 7.0
  │
  ▼
┌─────────────┐      Time:            2 minutes
│   Mixing    │      Speed:           maximum
└─────────────┘
  │
  ▼
┌──────────────┐     Time:            15 minutes
│Centrifugation│     Speed:           2000, 6000, 8000, 10000, 14000 xg
└──────────────┘
  │
  ▼
┌─────────────┐      Waste stream:    Insoluble fibres
│  Filtration │
└─────────────┘
  │
  ▼
┌─────────────┐      Time:            6 times 2 hours (12 hours total)
│   Dialysis  │      Pore size:       10 K MWCO
└─────────────┘
  │
  ▼
┌─────────────┐      Inlet temperature:   130 °C
│ Spray drying│      Aspirator pump:      90%
└─────────────┘      Pressure:            50 bar
  │                  Flow rate:           40%
  ▼
Fraction
```

Fig. 6. Schematic overview of all processing steps in sunflower seeds fractionation.

2.2.1.   Cake preparation

An oil press KK20 F Universal with a standard / soft seed screw (Figure 7) and a capacity of 20 kg / hour (Kern Kraft, Germany) was used to press the sunflower seeds. Sunflower oil was collected in a bucket and the press cake in a separate basket. Diameters of 1 and 1.5 mm and speeds of 11 and 22 RPM were studied in order to determine the optimum oil extraction conditions. Major caution was taken so that under these conditions the press would process the sunflower seeds at temperatures below 70 °C, which does not lead to a loss of advantageous native functional structures [39]. The cake that contained the least amount of oil was chosen for fraction preparation.

Fig. 7. Oil press and screw used during cold pressing.

2.2.2.   Fraction preparation

Milling

The cake fraction obtained after pressing was milled using a Pulverisette 14 (Fritsch, Germany). A sieve size of 2 mm and a speed of 8000 RPM were used to break the cake to receive particles that are better dispersible.

Dispersing

The cake particles were dispersed in water at a ratio of 1:10 (100 g or 300 g sample) and stirred at 2000 RPM with an IKA Eurostar digital Euro-St D (IKA, Germany) for 2 hours. During stirring the pH was kept constant (6.8 – 7.0) by using NaOH (1 M) and HCl (1 M, 37 % v/v). Subsequently, the homogeneous

solution was mixed for 2 minutes at 6000 RPM using a Thermomix TM31 (Vorwerk, Germany).

Centrifugation

The obtained mixture was centrifuged for 15 minutes in a Sorvall Lynx 4000 Centrifuge (Thermo Fischer Scientific, USA) to separate the fibres from the fraction. The different centrifugal forces studied were 3408, 5903, 6816, 7621 and 9017 RPM (2000, 6000, 8000, 10000 and 14000 xg). The pellet containing the fibres was considered waste. The supernatant was filtered using a dumpling cloth (GEFU, Germany). The filtrate was brought to a pH between 6.8 and 7.0 before further processing. The residue was considered waste.

Dialysis

Dialysis was set up to remove small components as salts, saponins and minerals from the filtrate. The dialysis membrane was boiled for 15 minutes in water with 1 wt% EDTA. Thereafter, the membrane was closed at one side and filled with filtrate. After closing the other side of the membrane, the tube was placed in a vessel with 10 L demi water. The water was replaced every two hours for six times (total time of 12 hours). After dialysis the sample was brought back to a pH between 6.8 and 7.0.

Spray dryer

The dry matter content of the dialysed samples was tested with an Electronic Moisture Analyser MA35 (Sartorius, Germany). When the dry matter was above 2% w/w the sample was spray dried by a Buchi B290 mini spray dryer (Buchi Labortechnik, Switzerland). The inlet temperature of the spray dryer was set at 120 °C to keep the outlet temperature below 70 °C in order to avoid protein denaturation. The aspirator pump was set at 90%, the pressure at 50 bar and the flow at 40% (400 ml / hour).

2.2.3. Composition analysis

Subsequently, a composition analysis of the cake was performed in order to judge the optimal diameter and speed that led to the lowest oil content in the cake. A composition analysis of the fractions was necessary to find the centrifugal force that led to the highest amount of protein and fat in the fraction.

Protein content and composition

The Dumas method (AOAC method 902.23) was performed with a Nitrogen Analyser (Thermo Fisher Scientific, USA) to determine the nitrogen content of all cakes and fractions. A conversion factor of 5.7 [52, 60, 62] was used to calculate the protein content.

Fat content

The fat content of the fractions was measured after fat extraction for 7 hours with petroleum ether in an Extraction System B-811 (Buchi Labortechnik, Switzerland) (AOCS-AOAC 999.02).

Ash content

Fractions were put in an AAF 11/7 Ashing Furnace (Carbolite, United Kingdom) for 5 hours at 550 °C to determine the ash content (AACC Method 08-01).

Moisture content

The moisture content of fractions was tested in an Electronic Moisture Analyser MA35 (Sartorius, Germany) (AACC Method 44-15A).

Phenol content

Fractions were stirred on a Mix 6 Stirring Plate (2mag AG, Germany) for 2 hours at 900 RPM with 80% (v/v) methanol and water solution at a ratio of 1:20 (w/v) to extract the phenolic compounds. A centrifuge step was carried out for 20 minutes at 10000 RPM (17217 xg) using a Sorvall Lynx 4000 Centrifuge (Thermo Fischer Scientific, USA). This was needed for a separation of the pellet and the supernatant containing the phenolic compounds. This extraction procedure was repeated 6 times and its supernatants were collected. High performance liquid chromatography (HPLC) was performed in a Dionex Ultimate 3000 chromatograph (Thermo Scientific, USA) with a phenol C18 column (Sigma Aldrich, USA). A mobile phase made with 30 v/v% acetonitrile (ACN) and 0.1 v/v% trifluoroacetic acid (TFA) was used during analysis. The flow rate was set at 0.75 ml / minute. Calibration curves of chlorogenic acid, caffeic acid and dichlorogenic acid were compared with the graphs of the fractions to find the amount of phenolic compounds present.

Particle size distribution

To determine the size of the components within the fractions the laser diffraction particle sizer Mastersizer 3000 (Malvern Instruments Ltd, United Kingdom) was used. Fractions were first dissolved in water to obtain a fat concentration of 0.5% and then brought to a pH between 6.8 and 7 using NaOH (0.1 M). To break up coalesced / flocculated oil bodies 0.5 wt% 2-mercaptoethanol and 1 wt% sodium dodecyl sulphate was added to separate oil bodies. A refractive index of 1.09 for oil bodies and a refractive index of 1.33 for water was used [52]. Measurements were performed in triplicates.

2.2.4. Structure characteristics

Confocal light scattering microscopy (CLSM)

The fractions were optically analysed using LSM510 confocal light scattering microscopy (Zeiss, Germany) with an objective c-apochromat 40x / 1.2 W Corr M27 (Zeiss, Germany). Nile red was mixed with ethylene glycol at a ratio of 1:100 v/v% and subsequently mixed with water (1:3 v/v%). A droplet of this resulting stain was put onto a 1 µ-Slide 8 Well ibiTreat (Ibidi, Germany). Thereafter, factions were put onto the stain until the powder had stopped floating in the stain. Subsequently the well was placed under the microscope. During analysing, two filters were used in channels 1 and 3 respectively (LP 650 and BP 535-590).

Scanning electron microscopy (Table SEM and Cryo SEM)

For table SEM an Ultra Smooth Carbon Adhesive Tab (EMS, USA) was put onto the Aluminium Pin-Type Mount 12.7 mm (Jeol, The Netherlands). Fractions were deposited onto the tabs and subsequently attached to a Standard Sample Holder (PhenomWorld, The Netherlands). The sample holder with the fractions was placed into the Phenom Pure Table Scanning Electron Microscope (PhenomWorld, The Netherlands).

During cryo SEM fractions were deposited on Ultra Smooth Carbon Adhesive Tabs (EMS, USA), which were attached with CCC Carbon Adhesive (EMS, USA) onto a Brass Leica Sample Holder (Leica, Austria). The holder was affixed to the Cryo-Sample Loading VCT 100 System (Leica, Austria) and simultaneously frozen in liquid nitrogen. The frozen holder was transferred to the MED 020/VCT 100 Cryo-Preparation System (Leica, Austria) at -92 °C. For removal of frost contamination

the samples were freeze dried for 5 min at -92°C and 1.3×10^{-9} bar. After sputter coating with a layer of 20 nm tungsten at the same temperature the sample holder was transferred into the field Magellan 400 Emission Scanning Electron Microscope (FEI, The Netherlands) at -120°C. The analysis was performed with SE detection at 2 kV and 6.3 pA. Automatic droplet tensiometry (ADT), was performed to investigate the properties of the fractions in an emulsion.

2.2.5. Interfacial properties

Automated droplet tensiometer (ADT)

Surface tension and interfacial elasticity of the fractions at the oil / water interface (O/W) were measured using an Automated Drop Tensiometer (Tracker, Teclis Scientific, France). Fractions were dissolved in water at a protein concentration of 0.01 wt% and mixed on a Mix 6 Stirring Plate (2mag AG, Germany) for 30 minutes at 900 RPM. The next step was filtration (Whatman prepleated qualitative filter paper, Grade 595 ½, Sigma-Aldrich, USA). After filtration, an optical glass cuvette 40 x 23.6 x 15 mm (Hellma Analytics, Germany) was filled with 25 ml of the filtrate. A stainless steel j-shaped G-20 needle (Teclis Scientific, France) was attached to a 1 ml syringe (SGE Analytical Science, Australia) filled with stripped oil.

The stripped oil was obtained by adding aluminium oxide to sunflower oil in a 1:2 v/v% ratio. This was needed to remove impurities, polar compounds and tocopherols that could interfere at the oil-water interface. The mixture was stirred on a Mix 6 Stirring Plate (2mag AG, Germany) for 24 hours at 900 RPM without light exposure. Thereafter, the mixture was centrifuged in a Sorvall Lynx 4000 Centrifuge (Thermo Fischer Scientific, USA) at 3408 RPM (2000 xg) twice to separate stripped oil from the sorbent. The collected stripped oil was stored in the dark at -20 °C [68].

The surface tension was measured for 3 hours at a constant droplet volume of 20 µl and a droplet area of 30 mm^2. Oscillations measurements with a range of frequencies (periods of 200, 100, 20 and 10 s) and with a constant amplitude of 5% were performed to calculate the storage modulus (E') and loss modules (E''). The storage modulus reflects the elastic energy stored in the surface and the loss modulus represents the viscosity of the surface.

3. Results and discussion

3.1. Composition analysis

The first step in the experimental part was to do a composition analysis for the processed cake and fractions.

3.1.1. Effect of cold pressing on cake composition

It was decided to continue with the cake processed at a press diameter of 1 mm and at a speed of 11 RPM, because it had the lowest average fat content. The composition of this cake is shown in Table 2. The oil content is 10 wt% higher than after conventional processes, which was expected with aqueous fractionation [40, 41]. Therefore, the cake is not comparable to that of industry. The fibres cover a big part of the total cake [5, 11]. This is due to including the hull in the production process, which results in more fibres being present in the cake as when using dehulled seeds [21].

Table 2. Composition cake processed with diameters of 1 mm and at a speed of 11 RPM.

Components (wt%)	Cake
Protein	21.1
Fat	14.0
Moisture	9.7
Ash	4.7
Phenols	1.8
Fibres	48.7

Commercial sunflower seeds were used for oil extraction. The used batch consisted not only of pure sunflower seeds, but also contained some impurities. Hulled sunflower seeds were used as it leads to a higher oil recovery [69]. To be as close to industry as possible the first priority of this research was to focus on maximal oil extraction as this is the main goal of current industry [33]. Therefore hulled seeds were used for cake preparation. Although, literature is not consistent in the use of hulled seeds. Many state that dehulled seeds lead to higher protein contents in the cake, which is advantageous to the composition of the cake [70].

3.1.2. Effect of centrifugal forces on fraction composition

To understand the influence of centrifugal forces on the composition of the fractions different forces were tested. Fractions were prepared at 2000, 6000, 8000, 10000 and 14000 xg.

Protein content

Figure 8 shows the relation of centrifugal force on protein content (wt%). It can be noted from the graph that the protein content of all fractions produced is around 60 wt% with minor fluctuations. Therefore it can be concluded that centrifugal force has no influence on protein content in the fraction.

Ahmed and Schmidt [71] analysed the relation of centrifugal force on protein content extracted from peanuts and soy beans at pH 7. For soy beans, the protein content after centrifugation at 2000 xg and 40000 xg was both around 60%. There was also no significant difference found in protein content extracted from peanuts. These findings correspond to the conclusion drawn in the previous paragraph. Proteins remain in the supernatant regardless of the applied centrifugal force.
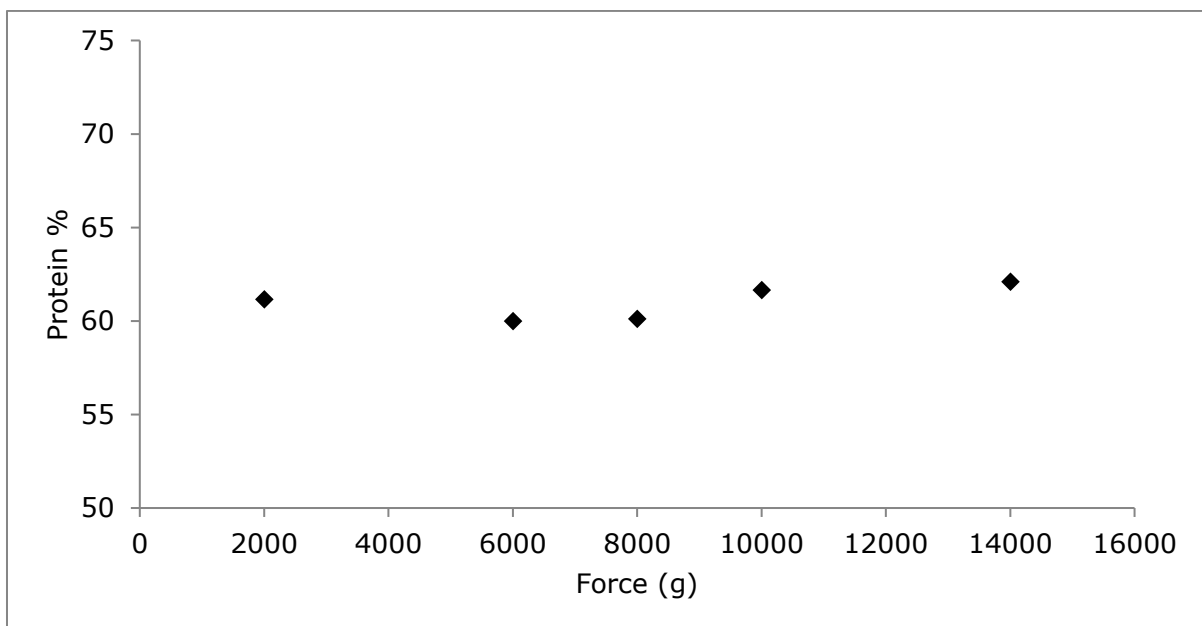

Fig. 8. Protein content (wt%) in fractions centrifuged at 2000, 6000, 8000, 10000, 14000 xg.

Lipid content

Figure 9 shows the fat content in the fractions at the centrifugal forces tested. As it can be observed in the graph, at low centrifugal forces the fat content is rather high, at about 20 wt%, but at forces higher than 8000 xg a sudden decline of more than 10 wt% fat was noticed thereby converting fat from a major to a minor ingredient in the fractions. However, this is very unlikely as it can be expected that at higher centrifugal forces a better separation of fibres and the soluble fraction is realised.
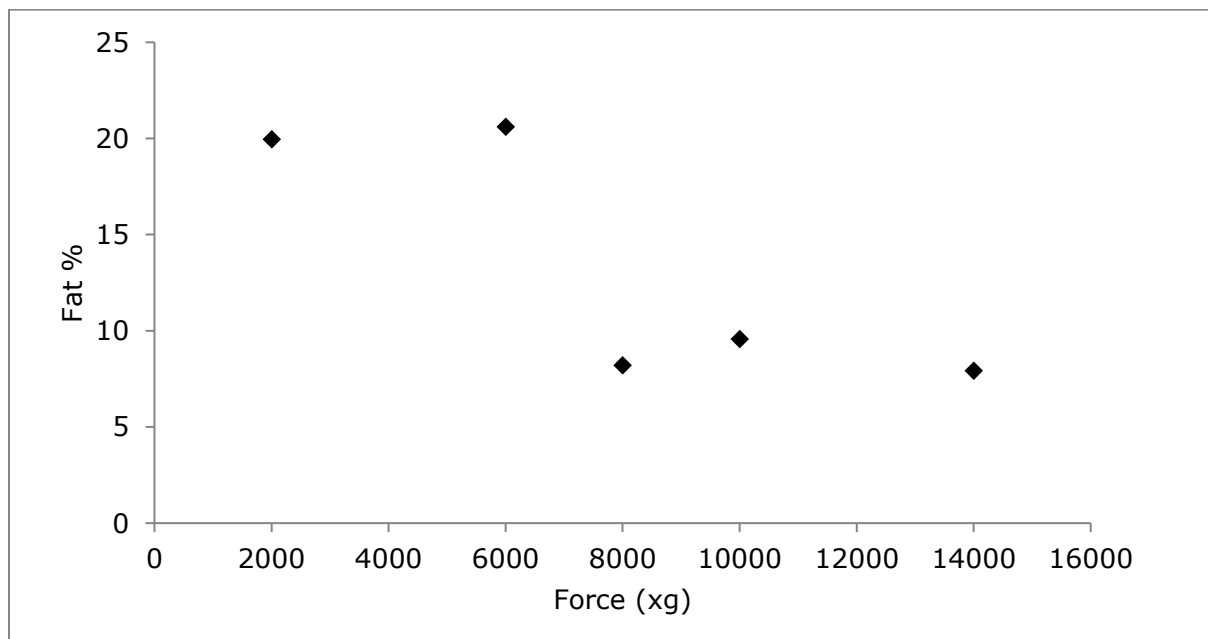

Fig. 9. Fat content (%) in fractions centrifuged at 2000, 6000, 8000, 10000 and 14000 xg.

Nour et al. [72] studied the effect of centrifugation force on oil extraction from coconut milk. At higher forces more oil was extracted. Oil droplets have a lower density than the other compounds and therefore move in the opposite direction of the centrifugal force. When the force is higher more oil droplets move to the surface and therefore more oil can be extracted. Nikiforidis et al. [36] also observed a cream layer after centrifugation of oil seeds. A cream layer was also observed during this research (Appendix A). A possible explanation of the sudden lipid decrease at forces higher than 8000 could be that the dense layer of cream was not able to pass through the filtration cloth anymore. Therefore, some lipids stayed behind and could not end up in the final fraction. This experiment needs to be repeated to confirm the sudden decrease in lipid content, because a more gradually decrease of lipid content should be expected. In addition, to be confident

in retrieving all lipids it is recommended to exclude the filtration step when preparing fractions in the future.

The full composition of the fractions can be found in Table 3. The variances of components present in the fractions centrifuged at 2000 and 6000 xg could be given as at this force two fractions were made. The fractions at 8000, 10000 and 14000 xg were only made once. The yield of fractions was the same at every force (5 wt% ± 0.5) regardless of the weight of the initial sample. Therefore, the centrifugal force had no influence on the yield of the fractions.

Besides lipids, all other measured components stay relatively the same when changing centrifugation force. This means that the decrease in fat content was accompanied by an increase in fibres present in the fractions. As fibres have a higher density as the soluble fraction they tend to move to the bottom of the centrifuge tube. Therefore, at higher forces, more fibres will be found at the bottom. This is contradictory to the results obtained in this research as the fibre content in the supernatant increases at higher forces. Structure analysis could possibly confirm this reasoning by comparing fractions centrifuged at 2000 and 6000 xg with fractions centrifuged at 8000, 10000 and 14000 xg. Differences in fibre composition could be visible. This will be discussed at the end of chapter 3.2.

Table 3. Full composition of fractions processed at 2000, 6000, 10000 and 14000 xg. Fibre content is obtained after subtracting the values of the other components from the total.

Components (wt%)	2000 xg	6000 xg	8000 xg	10000 xg	14000 xg
Protein	61.2 ± 5.2	60.0 ± 3.5	60.1	61.7	62.1
Lipids	20.0 ± 2.7	22.9 ± 2.3	8.2	9.6	7.9
Moisture	7.0 ± 1.3	6.5 ± 1.1	6.8	4.5	4.6
Ash	2.2 ± 0.6	2.5 ± 0.2	2.8	3.1	2.0
Phenols	2.3 ± 0.1	2.1 ± 0.2	3.8	3.6	3.6
Fibres	7.3	6	18.3	17.5	19.8

For structure and interfacial properties analysis the fraction obtained at 6000 xg is used as it has the most intriguing composition regarding protein and fat content (both rather high).

Particle size distribution

The size of the components within the fraction were analysed on particle size distribution with light scattering. In this case the size of the fat droplets was measured in order to compare the results with the average size of oil bodies so that it could be proven that the residual fat in the fractions was present mainly in the form of oil bodies. Possible aggregated fat droplets were first separated with the addition of SDS and 2-mercaptoethanol.

Figure 10 shows the size distribution of the dispersed fraction. The surface mean diameter varies between 0.7 and 10 µm with one big distinct peak around 1 µm and a smaller one around 8 µm. The first peak value indicates that the size of most fat droplets present is 1 µm.
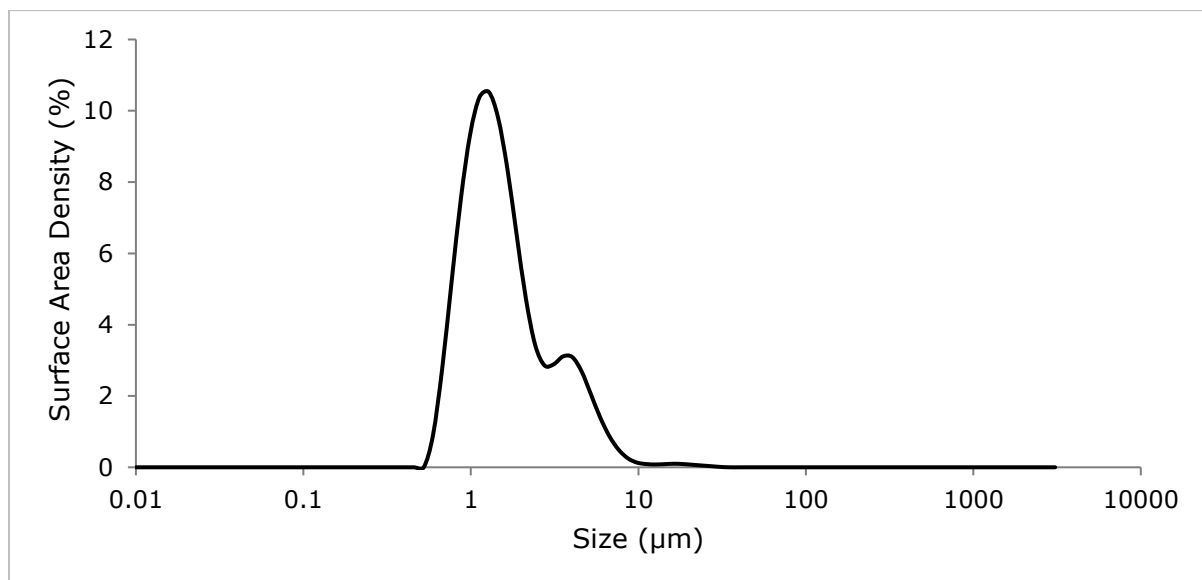

Fig. 10. Average size distribution of lipid particles present in a fraction.

The size corresponds to the size values of oil bodies found in literature [31, 52]. Ishii et al. [31] analysed the size of pure oil bodies (OB) and crude oil bodies (COB) extracted from soy beans. The crude oil body dispersion still contained some minor components as proteins. Both OB and COB had an average particle size around 0.8 µm. Nikiforidis et al. [52] analysed the particle diameter of sunflower oil body dispersions over time. At the start of the experiment the diameter of the particles was 8 µm, but when treated with SDS and 2-mercaptoethanol the diameter of the oil bodies was 1 µm. Oil bodies from Nikiforidis et al. were also analysed with cryo-SEM. The images show oil bodies with diameters around 1 µm. These findings correspond to the 1 µm peak in Figure 10.

With this information the small peak at 7 µm can be explained by some oil bodies still being aggregated. Adding more of these chemicals would probably result in one peak at 1 µm. Another explanation could be the presence of bigger oil droplets. Campbell [67] suspects an oil body disruption during processing. Cold pressing and aqueous fractionation might have resulted in disrupted oil bodies. Free proteins can stabilize the non-oil body lipids in dispersed droplets, which can have a bigger size than the original sunflower oil bodies. Figure 11 shows a schematic representation of what probably happened during cold pressing and aqueous fractionation. The upper oil body is cracked and the free flowing oil is quickly covered with free protein. This oil droplet might be bigger, which explains the second peak in Figure 10. The lower oil body survived the processing and therefore stays intact (peak 1). It can be suspected that after processing most lipids are present in oil body form, but some did not survive processing and are therefore stabilized in oil droplets.
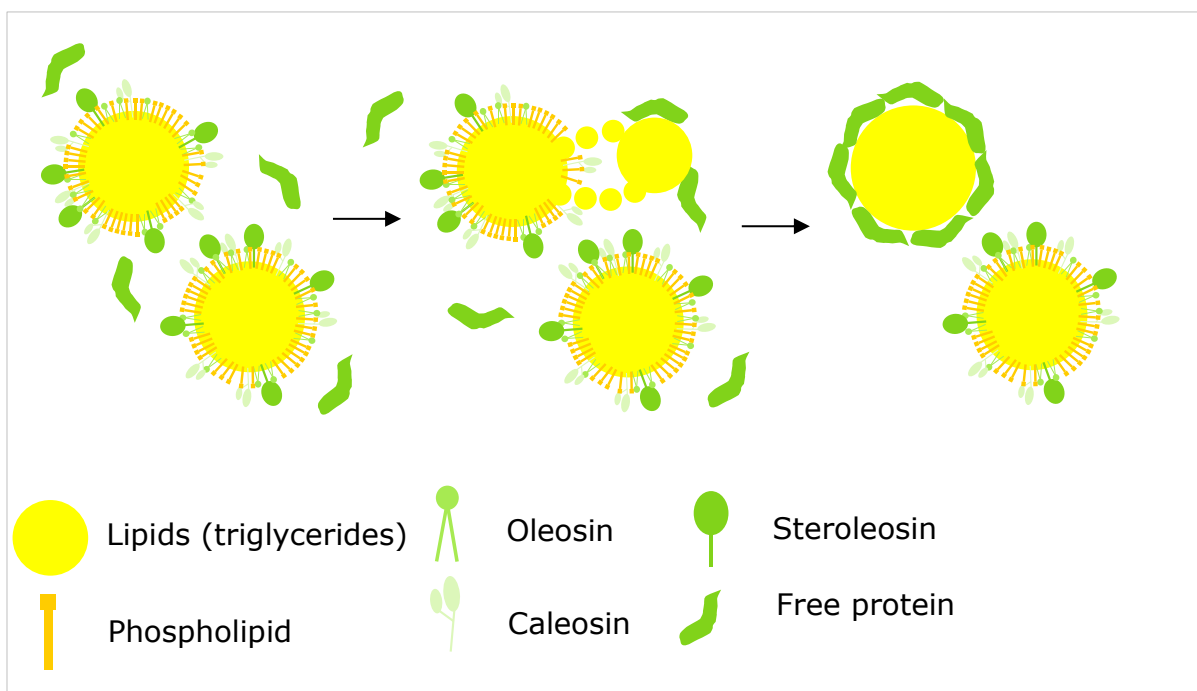

Fig. 11. Schematic representation of the rupturing of oil bodies during processing into oil droplets stabilised by free protein.

3.2. Structure characteristics

The second step in analysing the sunflower-based fractions was a structure analysis. Emphasis was placed on the lipid particles as it is not known if they are present in original oil bodies or in stabilized oil droplets.

3.2.1. Confocal light scattering microscopy (CLSM)

Figure 12.1 shows an image of a fraction stained with nile red. Nile red was used as a stain as this stain is soluble in lipid environments and exhibits fluorescence emission within the fat particles [53, 73]. Fluorescence emission of the lipid environment was recorded. Spherical shaped particles of sizes between 0.5 and 3 μm are clearly enlightened which points towards the direction of their identification as lipid particles.

This is supported by a CLSM image of a defatted fraction (Figure 12.2). The spherically shaped particles are missing, which leads to the conclusion that the spherical particles must be fat particles. The amorphous structures surrounding the fat particles in Figure 12.1 are also present in Figure 12.2. and probably represent proteins as they are the main component of both fractions. Extra support to this theory is given by another CLSM image (Figure 12.3), which shows a powder consisting of oil bodies. The proteins have been extracted prior to the CLSM analysis. No amorphous structures can be observed, which reinforces the explanation of the amorphous structures being proteins.

In addition, Nikiforidis et al. [52] and Gallier et al. [53] (Figure 12.4 and Figure 12.5) have published CLSM pictures of oil bodies as well. However, Nikiforidis et al. stained the oil body fractions with nile blue and Gallier et al. used nile red for the oil bodies and fast green FCF for the proteins. A similar structure can be observed of oil bodies surrounded by proteins. As their fractions consisted mostly of oil bodies the amorphous structures representing protein was not as apparent as in Figure 12.1 and 12.2, but it was still visible.
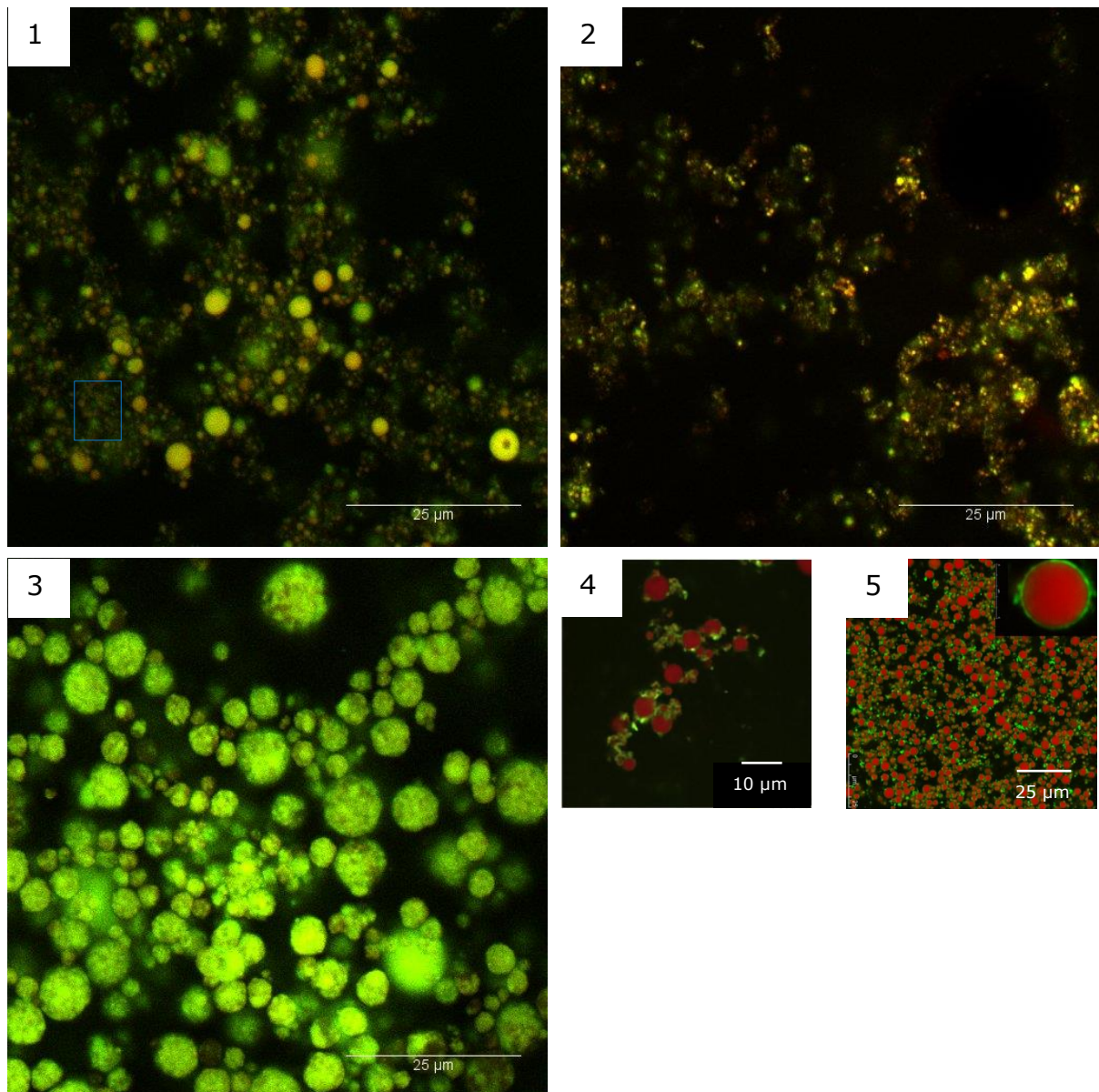
Fig. 12. 1: CLSM image of a fraction stained with nile red. 2: CLSM image of a defatted fraction after defatting stained with nile red. 3: CLSM image of rapeseed oil bodies (internal research Nikiforidis) stained with nile red. 4: CLSM image of sunflower oil bodies stained with nile blue [52]. 5. CLSM image of almond milk oil bodies stained, lipids stained with nile red, proteins stained with Fast Green FCF [53].

The size range of the spherical particles is comparable to the size of sunflower oil bodies found by Nikiforidis et al. [52] (Figure 12.4). As explained in the previous chapter Nikiforidis found the size of sunflower oil bodies to be around 1 µm. Hence, this does not mean that the spherical particles are indeed original oil bodies. Campbell [67] stated that processing could lead to disrupted oil bodies of which the lipids are encapsulated with free protein. Therefore the spherical particles in Figure 12.1 and 12.2. could also represent oil droplets formed during processing.

From Figure 12.3 it can also be noticed that all oil bodies have an average size around 3 µm. The difference in source between the lipid particles present in Figure 12.1 and 12.3 (sunflower versus rape seed) could explain the difference in size. Hence, Nikiforidis et al. [36]. explains that the size of the recovered oil bodies from the initial extract is determined by the extraction method and the presence of exogenous proteins. The amount of proteins in the oil body extract in Figure 12.3 is low and therefore the oil bodies are covered with less proteins than in Figure 12.1. This gives the lipids more space to form bigger particles. The different extraction method or the presence of proteins gives an explanation of the size difference. Still, these findings about the size do not tell anything about the lipid particles in the fraction being oil bodies or oil droplets.

Noticeable are the different colours of the spherical particles in Figure 12.1. The green colour (wavelengths 535 – 590 nm) is associated with low polarity and the orange colour (wavelengths larger than 650 nm) can be correlated with elevated levels of polarity. Figure 12.3. shows that the oil bodies have a green colour. This green colouring must be typical for oil bodies and therefore correspond to its polarity as explained before. It can be assumed that the green particles in Figure 12.1 are oil bodies that are still intact after aqueous fractionation. The orange particles consist probably out of oil droplets, which are probably of a different polarity and with that a different colour.

An explanation for different colours between the fat particles can be found on the interfacial layer of the particles where different kinds of proteins might be present or have absorbed. Sackett and Wolff [74] explains that the level of hydrophobicity of proteins results in different emissions. β-Lactoglobulin is a very hydrophobic molecule and has the highest intensity of fluorescence at wavelengths of 605 nm. Lysozyme is more hydrophilic and has an intensity peak at 650 nm. Sacket and Wolff state that the nile red dye is altered when it binds to different proteins. This would explain the different colours observed in Figure 12.1. The green particles are surrounded with hydrophobic proteins as the wavelengths correspond to the wavelengths found by Sacket and Wolff (around 600nm). The orange particles have a more hydrophilic layer of proteins as its wavelengths are higher than 650 nm.

Huang [75] found that oleosins are one of the most hydrophobic proteins found in plant materials. As oil bodies are covered predominately in oleosins (see Chapter 1.2.) it can therefore be suggested that the green particles are original oil bodies. The orange particles are covered with less hydrophobic proteins and might therefore be oil droplets covered with free proteins.

3.2.2. Scanning electron microscopy (Table SEM and Cryo SEM)

Scanning electron microscopy (SEM) was done to complement and clarify the findings of confocal light scanning microscopy. Two different types of SEM analysis were performed namely table SEM and cryo SEM.

Table scanning electron microscopy (Table SEM)

Table SEM was used as a straight forward easy technique to analyse the structure of the fraction in powder form (no hydration). Figure 13 shows images of the fraction at different magnifications. Immediate attention is drawn to the big spherical shaped particles in Figure 13.1. A magnification of the middle particle can be found in Figure 13.2. The size of the particle is around 100 μm, which is much bigger than the average size of an oil body (~1 μm).

An explanation for the appearance of these particles could be an aggregation of oil bodies or oil droplets before spray drying. During spray drying the particles stayed aggregated and became one big spherical particle. According to Maa et al. [76] spherically shaped particles are formed during spray drying. These big particles were not found in CLSM as no particles bigger than 3 μm were observed. Possibly these particles have dissolved in the stained water before being analysed by the CLSM. There was no pre-treatment when analysing particles with the Table SEM and therefore these particles could be observed.
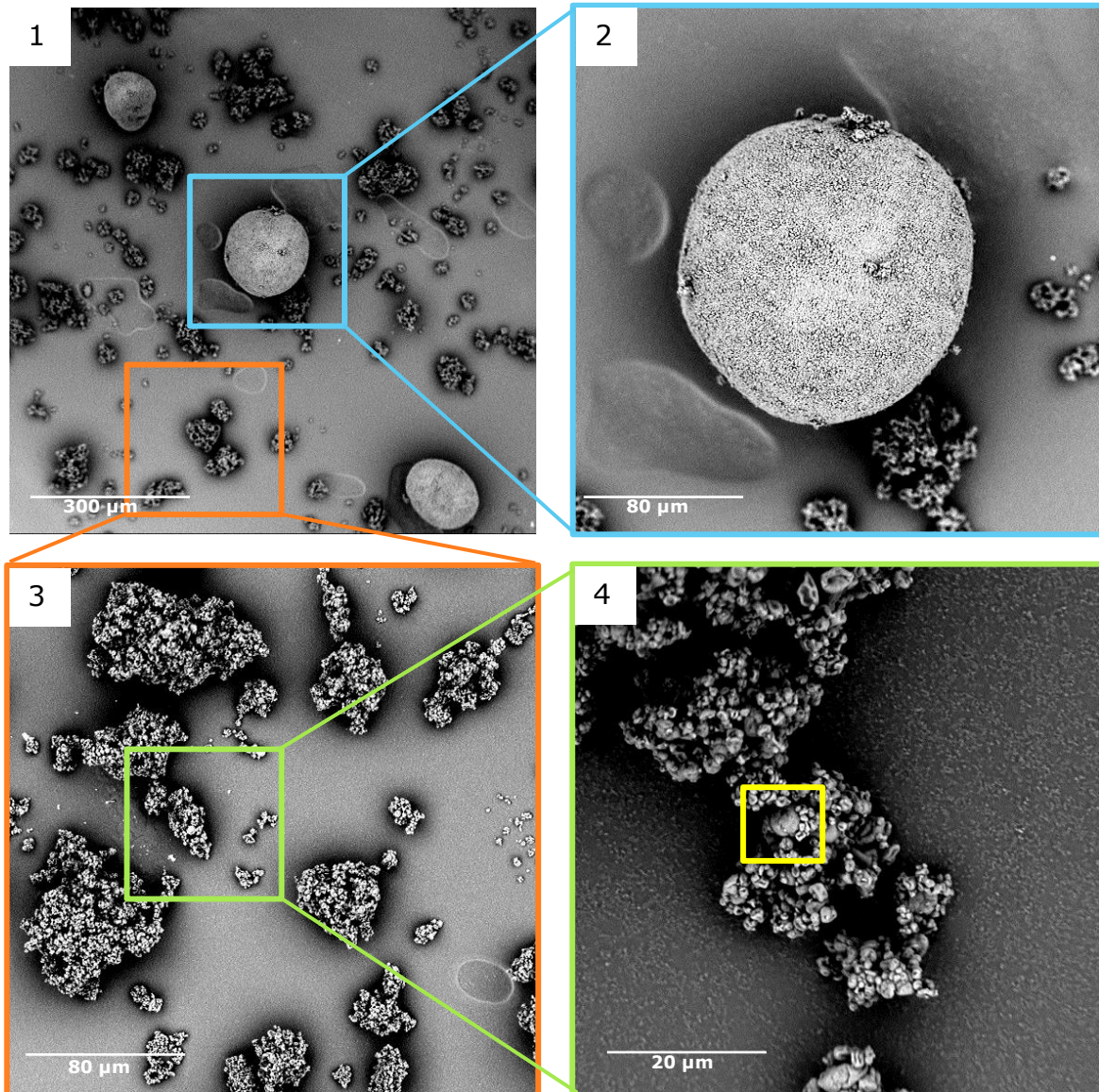
Fig. 13. Table SEM images of a fraction. (1: magnification 275x, 2 magnification 950x, 3: magnification 950x, 4: magnification 3500x).

Figure 13.3 zooms in on the other particles that are also visible in Figure 13.1. This image shows small particles clustered together, but no clear distinction between them can be made. Figure 13.4 is in higher magnification and a better distinction between particles can be observed. The particles are smaller than 2 μm and seem amorphous. Some spherical particles can be identified that have the same size as oil bodies (yellow rectangle in Figure 13.4).

To strengthen the conclusion regarding the structure of components present in the fraction, table SEM imaging was also done for a defatted fraction (Figure 14.1) and an oil body powder (Figure 14.3). These two images are compared at the same magnification (750x) to an image of the same fraction that was depicted in Figure

13. The image of the defatted fraction shows a scattering of very small particles. These particles must be the proteins, because that is the main component in the defatted fraction. The image containing the oil body powder (Figure 14.3) shows a fraction of particles clustered together. These particles are the oil bodies that built a network similar to the clustered particles in Figure 14.2. It becomes apparent that oil bodies are the components responsible for establishing a network between the compounds present in the fraction.
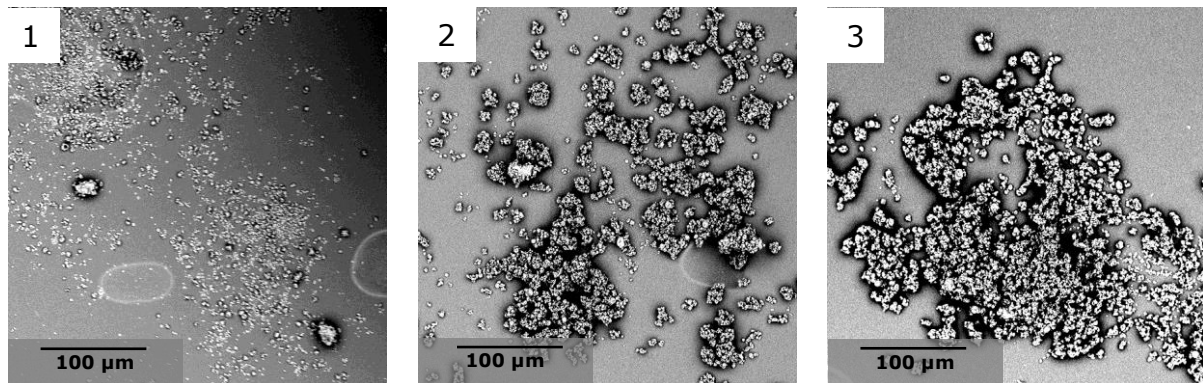

Fig. 14. Table SEM pictures of 1: a defatted fraction. 2: fraction. 3: a rapeseed oil body powder (internal research Nikiforidis). All at a magnification of 750x.

Cryo scanning electron microscopy (Cryo SEM)

A larger magnification is needed to get a better impression of the structure of the oil bodies itself as these table SEM images only show the network. Therefore cryo SEM imaging was done. Figure 15 shows images of the fraction at a 50000x magnification. Semi spherically shaped particles with a size around 2 µm are observed. In the first image (Figure 15.1) some small holes and punctures (white arrows) are present within the particles whereas in the second image the particles have a smooth surface (Figure 15.2).
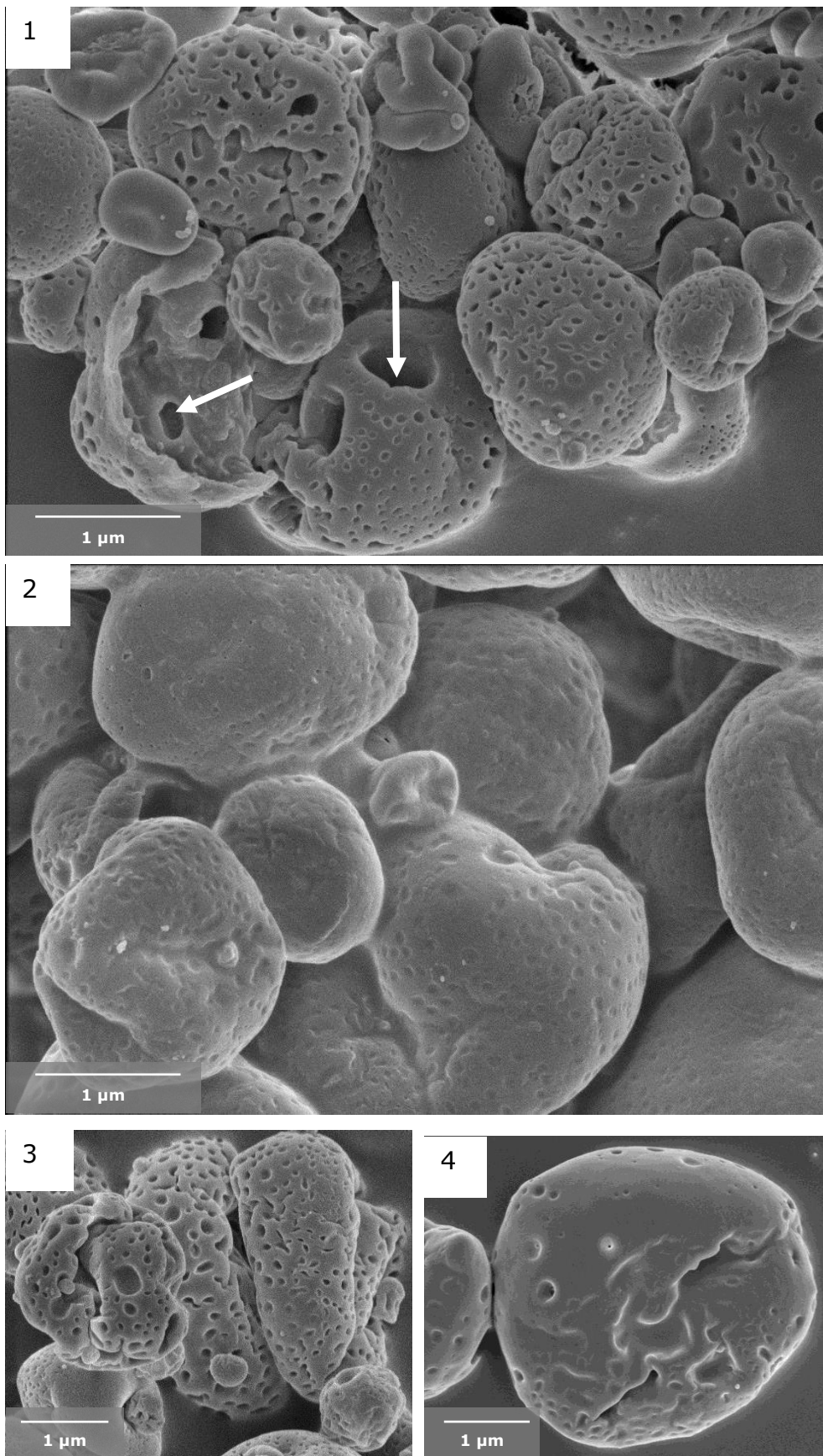
Fig. 15. 1, 2: Cryo SEM images of a fraction. 3: Cryo SEM image of a defatted fraction. 4: a rapeseed oil body powder (internal research Nikiforidis). All at a magnification of 50000x.

Cryo SEM images of a defatted fraction (Figure 15.3) and an oil body powder (Figure 15.4) were made to compare surface structures to those shown in Figure Figures 15.1 and 15.2. The images of the fraction show particles with a similar size and shape compared to the particles in the defatted fraction and the oil bodies. However, much more punctures and holes can be observed in Figure 15.1 and the defatted fraction compared to Figure 15.2. and the oil body powder.

A possible explanation to this is the cold pressing prior to analysis. Oil is extracted from the oil bodies by mechanical pressing. Therefore, some of the oil bodies are ruptured or punctured (Figure 15.1). Rosenthal et al. [77] explains that mechanical pressing only ruptures the membrane of oil seeds mildly, which explains that some of the oil bodies can still be intact. Figure 15.2. could therefore represent the intact oil bodies that are comparable to those presented in Figure 15.4, while Figure 15.1. shows oil bodies that did not survive processing. The latter is therefore comparable to the punctured particles presented in Figure 15.3. as all oil bodies were ruptured during harsh fat extraction with petroleum ether.

The findings of the structure analysis is in contrast to the statement of Campbell and Glatz [67, 78], which refers to the oil bodies of Nikiforidis and Kiosseoglou [60] as not being native oil bodies. Nikiforidis and Kiosseoglou produced oil body creams from maize germ with aqueous extraction at different pH values. Campbell states oil bodies do not survive the conditions during aqueous fractionation (pH 8-9, temperature 50 °C) as the interfacial surface of the cream only consisted of minor quantities oleosin, the primary protein present at the oil body surface. The mixture of interfacial proteins that Nikiforidis and Kiosseoglou found is not due to absorption of proteins at the native oil body interface, but is a result of oil body disruption and coalescence during processing. In contrast to the statement of Campbell and Glatz that no oil body survives aqueous fractionation, in this research a safe conclusion can be made that after aqueous fractionation a part of the fraction still consists of native oil bodies. CLSM showed that some lipid particles had a very hydrophobic environment, probably due to oleosins present on the surface indicating these particles are oil bodies. The CLSM images showed that fractions also contained some particles with a more hydrophilic environment. These particles are probably the disrupted and coalesced oil droplets that Campbell and Glatz were aiming at.

With the CLSM, table SEM and cryo SEM a clear structure analysis of the fraction was made. A safe conclusion can be made regarding the definite presence of oil bodies in the fractions. As some of the particles are not ruptured it can be concluded that some oil bodies are still intact.

Next to the pictures made of the fraction centrifuged at 6000 xg, the other fractions were analysed in order to investigate the differences in fibre content. This was needed to confirm that fibre content increases at higher centrifugation forces, a phenomenon which could not be explained in Chapter 3.1.2. A comparison of the fractions centrifuged at different forces is done with CLSM and table SEM imaging and can be found in Appendix B and C.

No difference was found between the samples. In all fractions fibrous structures were found (Appendix D). Images of sunflower seed fibres obtained from the sunflower cake are included as a reference. These findings implicates that CLSM and table SEM imaging are not qualifying methods to quantify the amount of fibres present. Therefore, it cannot be concluded that the fibre content is increasing at higher centrifugal forces. What happens with the fractions when being centrifuged at forces higher than 8000 xg remains unclear. To prove that this contradictory phenomenon is actually happening the fraction procedure and component analysis should be repeated.

3.3. Interfacial properties

To identify the interfacial properties of the fraction automated droplet tensiometry was performed. The properties of the fraction at the interface implicate the possible applications for functional protein fractions.

3.3.1. Automated droplet tensiometer (ADT)

The emulsifying ability of materials depends on their tendency to absorb on the oil water (O/W) interface. When materials absorb to the interface the surface tension of the emulsion decreases resulting in the formation of an emulsion. At lower surface tensions the emulsion is more stable. The decrease in surface tension was measured with an automated droplet tensiometer (ADT).

Figure 16 shows the surface tension of a fraction and a defatted fraction measured over a time period of 3 hours at a constant droplet area. This comparison was made to observe how the presence of lipids influences the surface tension of the

O/W interface. The green graph represents a fraction consisting of proteins, oil bodies and phenolic compounds and the grey graph corresponds to the same fraction, but defatted. Assumed is that there are no surface-active molecules present in the stripped oil and MilliQ water. The interfacial tension between water and sunflower oil with no present surfactants is 28 mN/m [31].

Figure 16 shows that for both fractions the surface tension initially decreases rapidly, in a few hundreds of seconds, from 28 mN/m to about 12mN/m and then reaches a constant value. It can be observed that the green line stays beneath the grey line in the beginning of the graph, which implicates a faster absorption of small molecules to the interface. This implicates that the material present in the fractions faster absorbs to the O / W interface than the material in the defatted fractions.
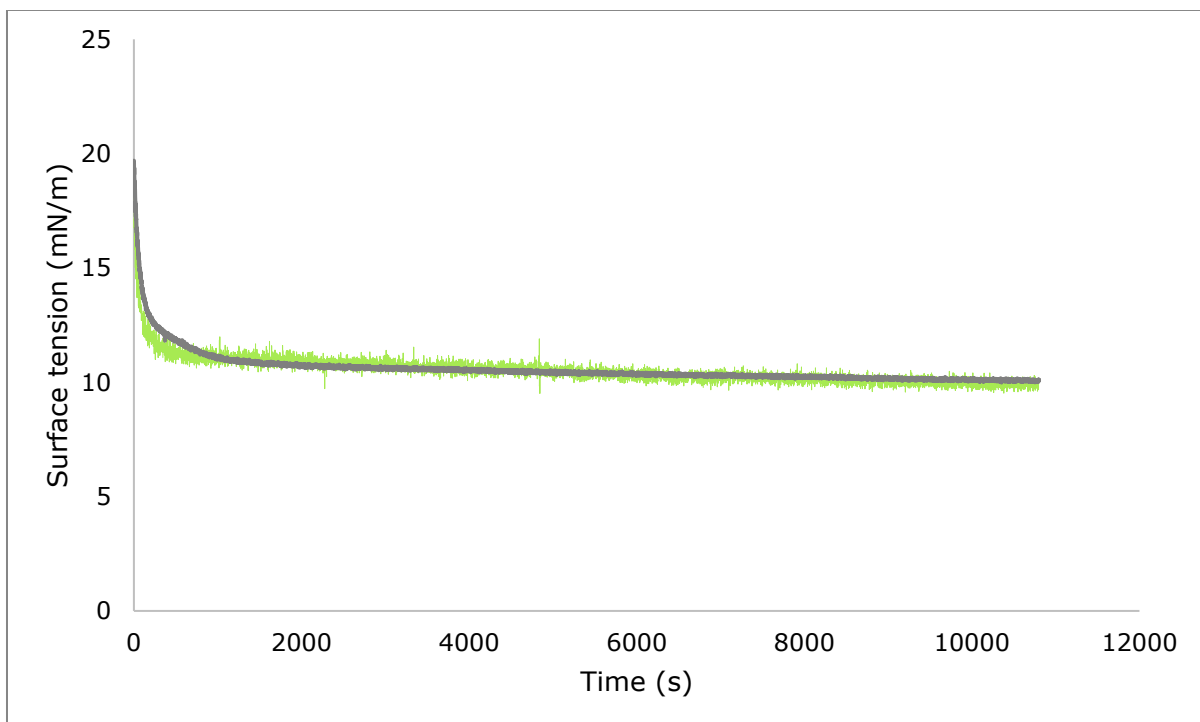

Fig. 16. Surface tension at the oil / water interface of a fraction (green) and a defatted fraction (grey).

When the fractions reach constant values the grey line is still above the green one. At this point the absorption has reached the equilibrium stage. At the equilibrium stage the surface tension of the defatted fraction is higher than the surface tension of the normal fraction. Although, it should be mentioned that the differences in surface tension between the normal and the defatted fraction are rather small. The

absolute value of the surface tension of the fraction and defatted fraction is around 10 mN/m (Figure 16).

Literature supports the positive contribution of oil bodies to the stability of emulsions [31, 32]. As mentioned in the chapter about particle size distribution Ishii et al. [31] analysed the size of pure oil bodies (OB) and crude oil bodies (COB) extracted from soy beans. Ishii (2017) [31] found the surface tension for OB to be around 12 mN/m, which is much lower than 28 mN/m. This indicates that oil bodies positively contribute to the stability of the O / W interface. However, Deleu et al. (2010) [32] also makes a logical statement that smaller particles move easier to the surface than bigger particles do. A faster absorption of the defatted fraction should be expected as during defatting the biggest particles namely the oil bodies were extracted. But, as chapter 3.2 shows the defatted fractions still contain the structures of the oil bodies only without the oil being inside. These structures can interfere with the smaller proteins wanting to absorb at the interface leading to a higher surface tension. This again, implicates the absorbing capacity of oil bodies as they do not interfere with the proteins, as the surface tension is almost the same. It can even be suggested that oil bodies interacting with proteins decrease the surface tension even more as the value for surface tension is a bit lower.

The equilibrium value of 10 mN/m is rather high compared to values found in literature. Karefyllakis et al. (2017) [26] found a surface tension around 5 mN/m for sunflower protein isolates (SFPI) mixed with CGA. This SFPI-CGA fraction is similar to the fraction in Figure 16 only with the lack of oil bodies. This would implicate that the presence of oil bodies results in a higher surface tension. Oil bodies would therefore not be beneficial for the stability of the emulsion. Ishii (2017) [31] found the surface tension for ideal oil bodies to be around 12 mN/m, which is indeed higher than that of proteins together with CGA and higher than the fraction that also contains proteins and other small components. The proteins in the fraction seem to contribute to the decrease of the surface tension. Although it should be mentioned that the fractions Karefyllakis et al. (2017) [26] and Ishii (2017) [31] used to find the surface tensions were rather ideal. Using a mixture of SFPI with CGA or pure oil body powder is not a reliable comparison to a fraction obtained after an applied process as aqueous fractionation. Therefore the surface tension value found for a fraction obtained after applied processing is rather promising.

To determine the viscoelasticity of the fractions the oil droplet area contacting the fraction solution was oscillated at an amplitude of 5% and at frequencies sweeps with periods of 0.005, 0.01, 0.05 and 0.1 s. The measurements were started after 3 hours when an equilibrium was reached of constant surface tension. Figure 17 shows the elastic modulus (E') and the loss modulus (E'') obtained by these measurements plotted against frequency. For both fractions the storage (elastic) modules was higher than the loss (viscous) modulus. Therefore, it can be concluded that the O/W interface behaves rather elastic than viscous. For the interface made with the fraction the elastic modulus was higher (18 mN/m) than for the interface made with the defatted fraction (17 mN/m). This indicates that the presence of oil bodies in the fraction strengthens the network surrounding the droplet.
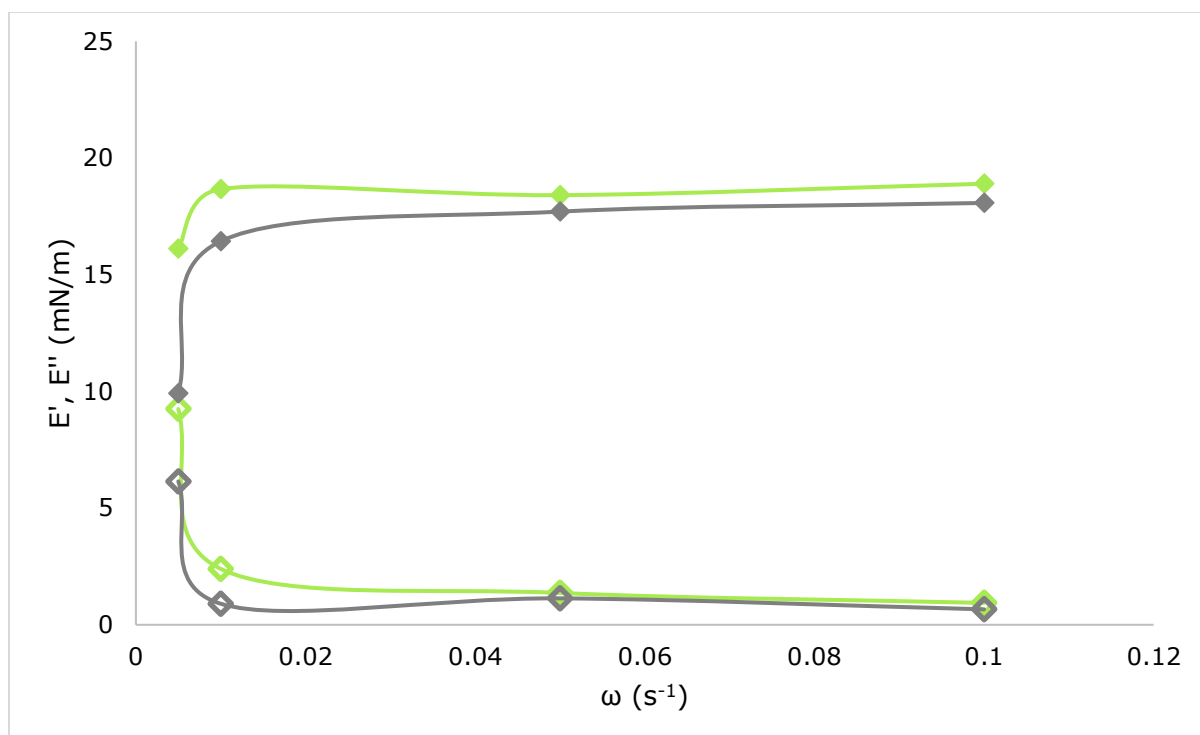

Fig. 17. Elastic modulus (closed symbols) and loss modulus (open symbols) for fraction (green) and defatted fraction (grey).

Karefyllakis et al. [26] had the same result for protein-CGA complexes compared to PI. The elastic modulus of the protein-CGA complex (1:1) was 5 mN/m higher than that of PI. The findings of Karefyllakis, but also the findings in this research indicate that fractions result in more protective networks surrounding the oil droplet. This fraction network has an elastic behaviour, which suggests that the interactions between proteins, oil bodies and phenolic compounds are good in maintaining the network surrounding the droplet. This network is resistant against oscillation and can form stable emulsions. This makes the use of fractions for food applications very promising.

4. Conclusion

The aim of this research was to gain more insights into sunflower-based protein fractions for food emulsions. In order to achieve this sunflower seeds were cold pressed and fractions were produced mildly through aqueous fractionation. The composition of the cake was analysed to find the cake with the lowest oil content during pressing. Secondly, the composition of the fraction was analysed to find the influence of centrifugal force during aqueous fractionation on the composition of the obtained fractions. The structure was analysed to indicate how the fractions with proteins as the main ingredient and lipids as a secondary ingredient are structured. Lastly, the interfacial properties were characterized to gain more insights into the role of other ingredients besides proteins within the fraction on the stability of the emulsion.

No direct relation on protein content was recorded while producing fractions with different centrifugation forces. An unexpected result was a sudden decrease in fat of 10 wt% at centrifugal forces larger than 8000 xg. Probably this was due to the filtration step were creamed lipid particles were not able to pass through the membrane. Structure analysis showed a network between proteins and lipid particles within the fraction that was absent after the removal of fat. It was proven with CLSM and SEM that part of these lipid particles are native oil bodies after comparing the images with oil body powder and defatted fractions. A characterisation of the interfacial properties revealed oil bodies within the fraction strengthen the stability of an oil in water emulsion. The value for interfacial tension was lower and the value for elastic modulus was higher for fractions compared to defatted fractions.

With these conclusions more insights into sunflower-based protein fractions for food emulsion is obtained. Utilizing sunflower cake for human consumption with aqueous fractionation is a good starting point for industry. Unknown is still the best process conditions for making fractions. But, before more research is done on that topic it should be determined what the best oil and protein composition is within fractions used for food applications. Because, next to maximum oil extraction, the quality of the end fraction should now also be taken into account as this is of high value as well.

5. Recommendations

Multiple recommendations can be given as this research was there to give more insights into sunflower-based protein fractions for food emulsions. More insights are definitely needed before industry will implement the use of functional instead of pure materials. First the recommendations to improve this research will be named and subsequently the recommendations for the next steps in this interesting field.

Current research

The experiment about the effect of centrifugation force on lipid and fibre content has to be repeated. In this research lipid content decreased suddenly at forces higher than 8000 xg accompanied by an increase of fibre content. The decrease in lipids can be explained by the filtration step as possibly the cream layer cannot pass this membrane. An explanation for the decrease of fibres could not be found. Therefore it is recommended to take a larger sample size when analysing the relation between centrifugal force and fraction composition. In addition, the filtration step could be excluded in order to be confident in retrieving all valuable lipids in the fraction.

If fibres seem to be increasing at higher centrifugation forces it is important to investigate the role of carbohydrates on structure and interfacial properties as well. These components might have an influence on the possible applications for sunflower-based fractions.

Multiple centrifugation steps comparable of those proposed by Geerts et al. would be interesting as well as this probably results in more valuable components ending up in the supernatant. A composition analysis of the pellet after centrifugation would indicate the losses of protein, fat and phenolic compounds.

Subsequently it would be interesting to analyse the structure and interfacial properties of the fractions before spray drying. Spray drying is energy intensive and exclusion of this step would reduce the impact on the environment. The use of fraction dispersions (instead of powders) was studied in previous research for yellow peas and could be interesting for sunflower seeds as well.

As is previously mentioned a blank oil in water emulsion during automated droplet tensiometry is needed to ensure no surface-active molecules are present in the sunflower oil and MilliQ water. When analysing fractions on emulsification properties in the future this should be taken into account. In addition, it would be interesting to analyse the behaviour of the fractions at the O / W interface a bit more. It is not known if oil bodies stay intact at the interface or burst open.

Future steps

Future research should study the most optimal fraction composition possible in relation to food emulsions. SFPI, pure oil bodies and phenolic compounds can be combined to find the optimal combination. With that information it can be recalculated what the composition of the cake must be to obtain the most optimal fraction. Subsequently, it will be known how much oil must be extracted to obtain the cake resulting in the most optimal fraction. It would be interesting to test if the use of dehulled seed results in better fractions and a higher overall yield of oil and fraction in comparison to hulled seeds.

An exergy analysis is needed to confirm the hypothesis that the use of cold pressing and aqueous fractionation has indeed less impact on the environment than the conventional process. This research has shown aqueous fractionation is a sustainable substitute to the conventional process. But, still some waste is produced of which the pellet after centrifugation is an example. A certain purpose for this fibre containing pellet should be found to make this a closed system.

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Appendix

A

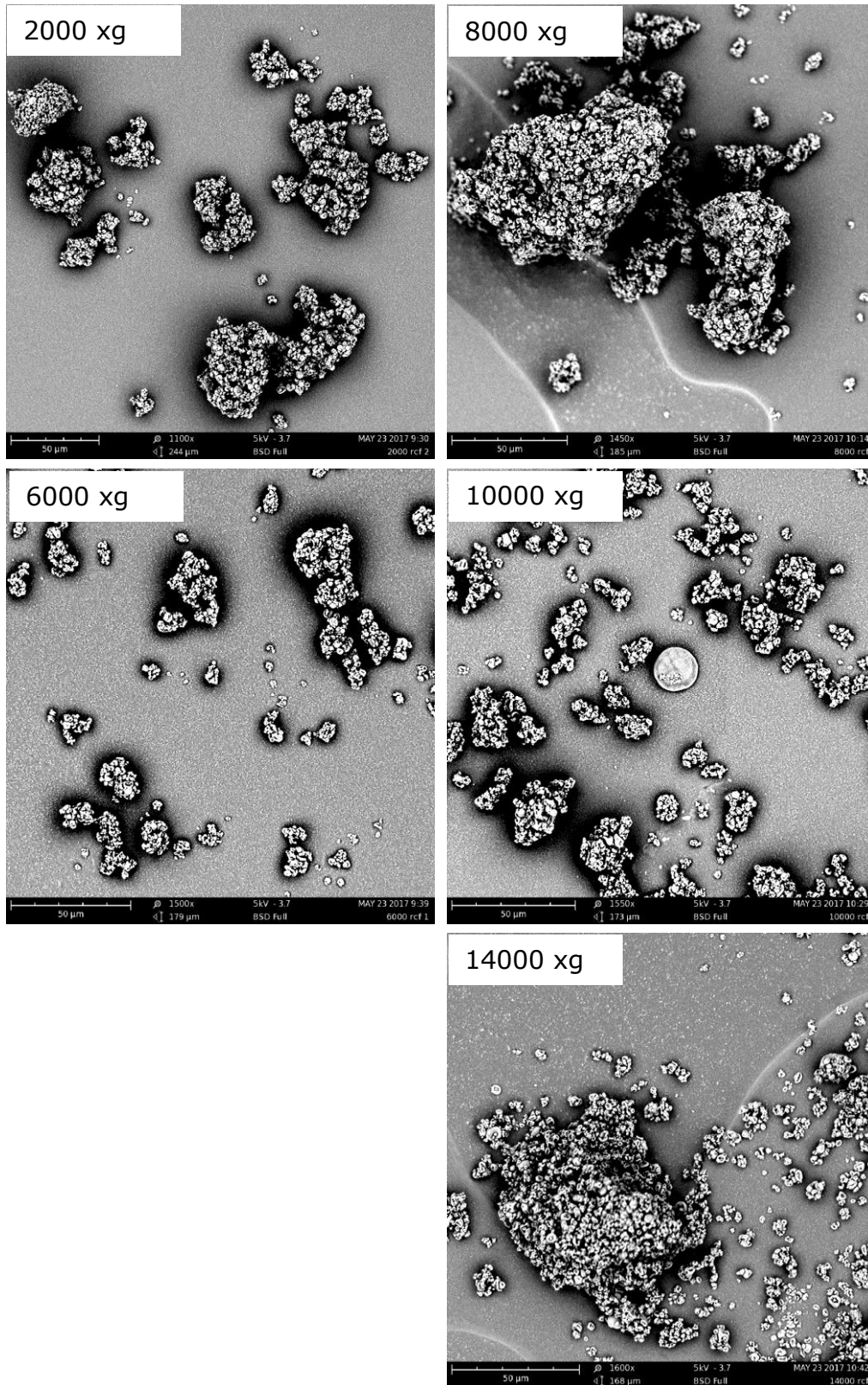**Fig. 18.** Cream layer after centrifugation.

B

Fig. 19. Table SEM images of fractions centrifuged at different forces (magnification 1500x).
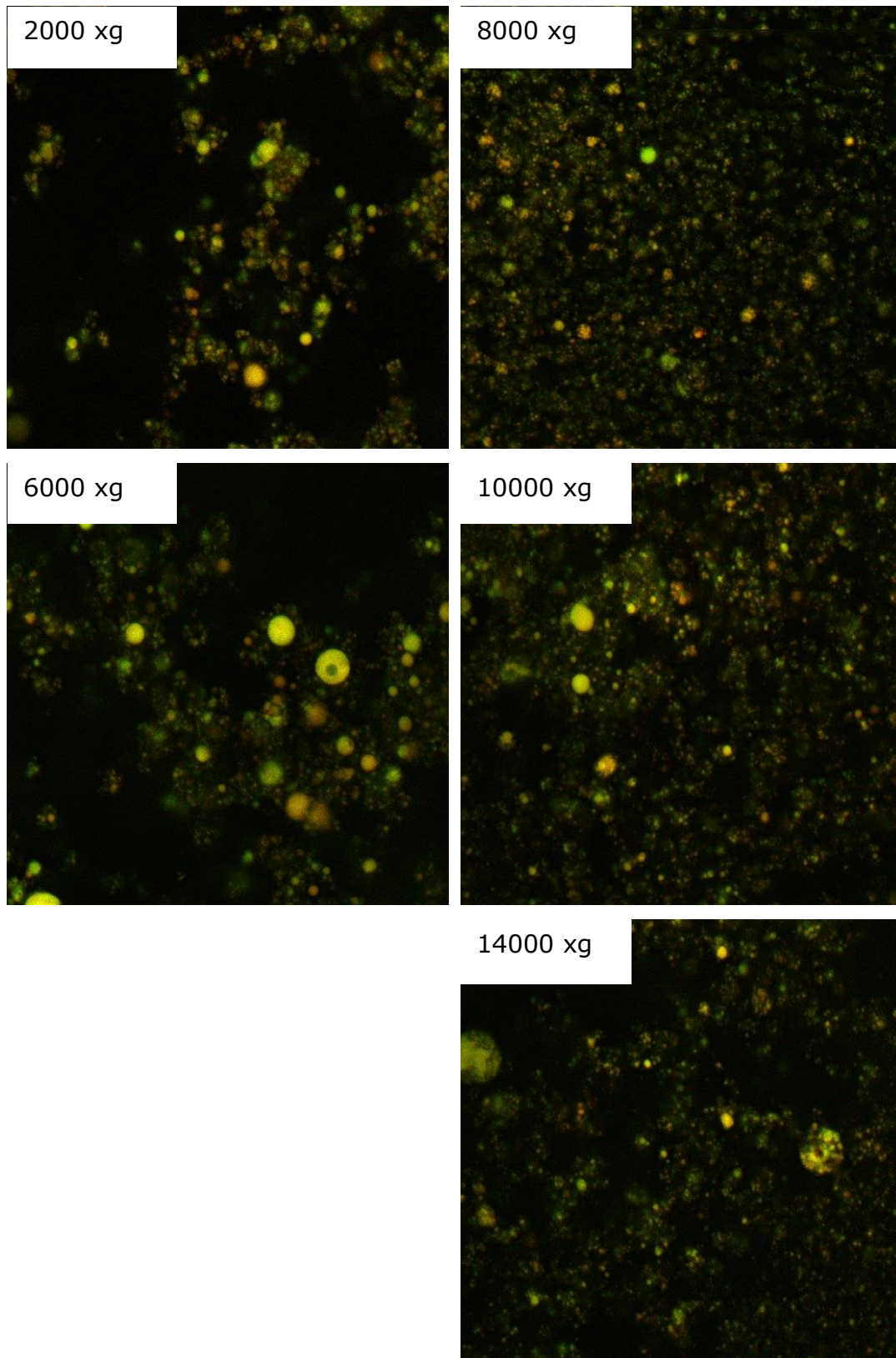
C

Fig. 20. CLSM images of fractions centrifuged at different forces (dimensions: 76.78 x 76.78 μm).
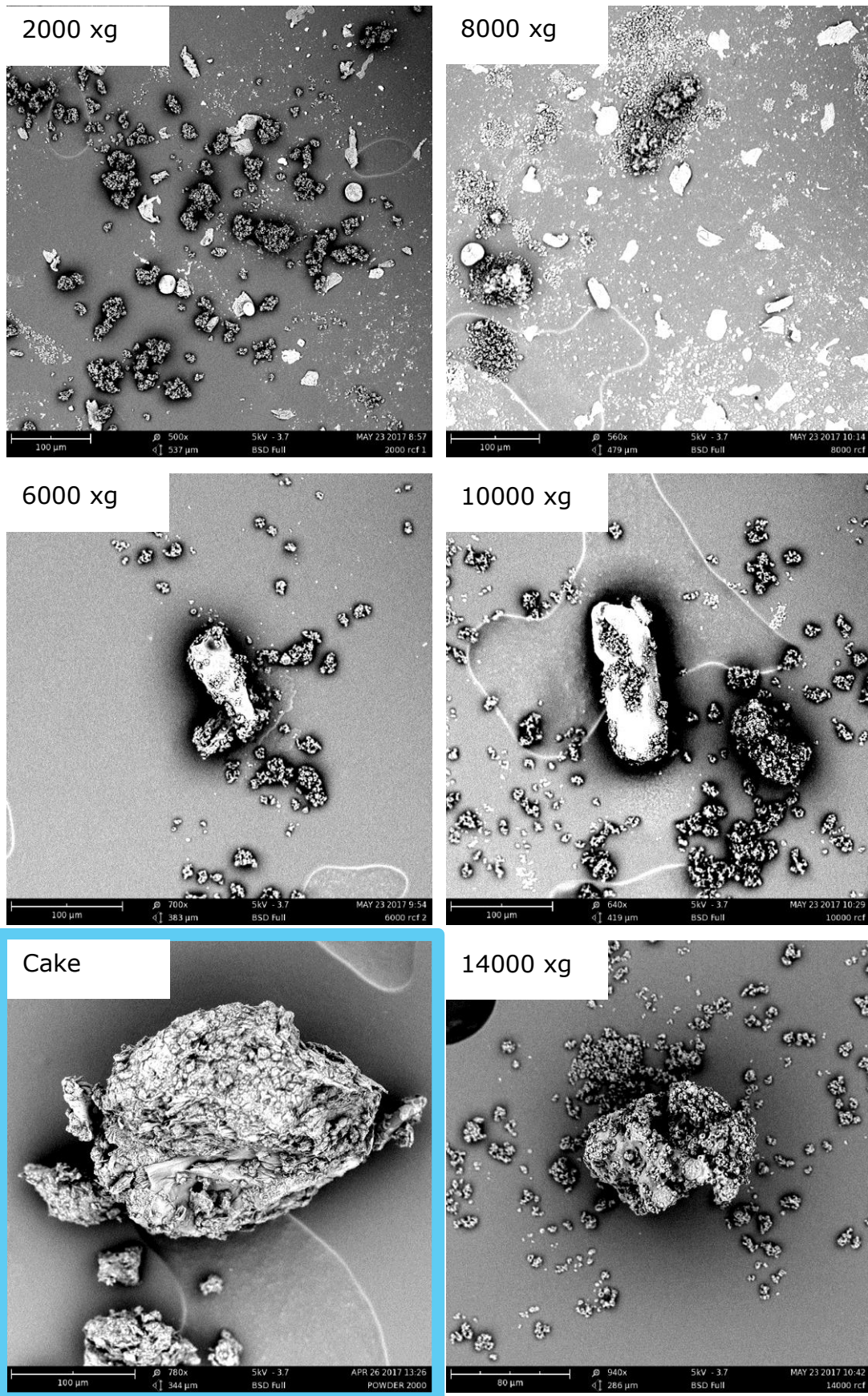
D

Fig. 21. Table SEM images of sunflower cake (blue framed image) and fractions centrifuged at different forces. Images are not at same magnification as this is not needed when only proving the presence of fibrous structures.